#7

Dkt. 56376/JPW

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Douglas A. Craig	
Serial No.:	09/450,880	Group Art Unit: 1655
Filed :	November 29, 1999	Examiner: Frank W. Lu
For :	Use of Compounds Which Activate A 5-HT Receptor To Treat Urinary Incontinence	

1185 Avenue of the Americas
New York, New York 10036

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

DECLARATION OF CARLOS FORRAY PURSUANT TO 37 C.F.R. §1.132

I, Carlos Forray, hereby declare as follows:

1. I am a Director of Cellular Sciences, Automation and Information Technology at Synaptic Pharmaceutical Corporation, the assignee of record of the subject invention. I hold a Medical Doctor degree and have practiced pharmacology for more than 25 years. A copy of my curriculum vitae is attached hereto as Exhibit 1.

2. I have reviewed the Final Office Action issued by the United States Patent and Trademark Office on December 21, 2001, wherein the pending claims 1-24 directed to methods of treating urinary incontinence in a human were rejected as not enabled by the specification of the subject application. I have additionally reviewed the subject application.

3. I understand that claims 1-24 are being rejected under 35 U.S.C. §112 in part because the Examiner alleged that the specification, while being enabling for treating murine

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urinary incontinence with compound 1 and compound 2 which activate the human 5-HT_{1F} receptor in an *in vitro* experiment, does not reasonably provide enablement for treating any kind of subject suffering from urinary incontinence by administering a therapeutically effective amount of a 5-HT_{1F} receptor agonist which activates the human 5-HT_{1F} receptor and can treat one subject suffering from urinary incontinence.

4. In the subject application the effects of 5-HT_{1F} selective compounds on the micturition reflex were assessed by their ability to inhibit the distension-induced rhythmic contractions of the rat bladder (the DIRC model). (See the specification as originally filed on page 22, line 25 through page 24, line 8.) The DIRC model is a rat *in vivo* model that is widely considered by those skilled in the art to be predictive for the activity compounds will have in treating human urinary incontinence (sometimes referred to as detrusor instability or unstable bladder). Urinary incontinence is the involuntary passage of urine from the bladder. I note that underlying conditions that result in what is clinically known as incontinence include overactive detrusor, overactive bladder, and interstitial cystitis. I also note that the DIRC model is also referred to as the volume-induced contractions of rat urinary bladder model.

5. In support of my statements in paragraph 4 above, that it is well-known to those skilled in the art that the DIRC model is predictive for the efficacy of compounds to treat urinary incontinence in humans, I note the teachings of the prior art. As described in Pietra, C., et al. (Effects of some antidepressants on the volume-induced reflex contractions of the rat urinary bladder: lack of

correlation with muscarinic receptors affinity (1990) *Pharmacological Research*, 22(4): 421-432, a copy of which is attached as Exhibit 2), the prior art teaches that the micturition reflex pathway is the same for both animals and humans and that the micturition reflex can be monitored *in vivo* by measuring the volume-induced contractions of the rat bladder. Pietra et al. (Exhibit 2) state on page 422, lines 2-6 that "[i]n both animals and humans micturition is initiated and maintained through the activation of a supraspinal vesicovesical micturition reflex pathway which can be monitored indirectly by recording the rhythmic, large amplitude intravesical pressure waves which occur when the bladder is distended and maintained under constant volume conditions".

6. It was well known to those skilled in the art prior to November 29, 1999 that compounds could be tested in the DIRC model to predict the activity compounds will have in treating human urinary incontinence. One example, PCT International Publication No. WO 97/31637 (Use of 5-HT_{1A} Antagonists for the Treatment of Urinary Incontinence, published September 4, 1997, a copy of which is attached hereto as Exhibit 3), teaches that 5-HT_{1A} antagonists were tested in the rat volume-induced contractions model to determine the predictive efficacy of 5-HT_{1A} antagonists for the treatment of urinary incontinence. PCT International Publication No. WO 97/31637 (Exhibit 3) states on page 7, lines 3-5 that the described rat *in vivo* models "were originally used to validate the predictive qualities of the true serotoninergic 5-HT_{1A} receptor antagonists for the foregoing urinary tract disorders."

7. Examples of drugs that are active in this model and are

also used therapeutically in humans to treat urinary incontinence were well-known to those of ordinary skill in the art as of November 29, 1999. One example, Morikawa, K., et al. (Inhibitory effect of inaperisone hydrochloride (inaperisone), a new centrally acting muscle relaxant, on the micturition reflex (1992) *European Journal of Pharmacology*, 213: 409-415, a copy of which is attached hereto as Exhibit 4), teaches that baclofen, a compound known to be "clinically useful for the treatment of patients with unstable bladder symptoms", and oxybutynin, a compound known to be "clinically useful for the treatment of detrusor instability", are active in the DIRC model. (See Morikawa et al. (Exhibit 4) on page 409, column 1, paragraph 1, lines 6-7; and page 409, column 2, lines 15-16.)

8. A second example, Guarneri, L. et al. (Effects of drugs used in the therapy of detrusor hyperactivity on the volume-induced contractions of the rat urinary bladder (1993) *Pharmacological Research*, 27(2): 173-187, a copy of which is attached hereto as Exhibit 5), teaches that nifedipine and terodiline, "drugs most commonly utilized in the therapy of overactive detrusor" are active in the DIRC model. (See Guarneri et al. (Exhibit 5) page 173, paragraph 1 of the summary, lines 1-2.)

9. A third example, Pietra et al. (Exhibit 2) teaches that imipramine and nortryptyline are active in the DIRC model. Pietra et al. (Exhibit 2) states on page 422, lines 7-8, that "[t]ricyclic antidepressants, particularly imipramine, have come to be accepted for the treatment of enuresis and a number of other micturition disorders." Enuresis is incontinence (involuntary passage of urine) that occurs at

night or during sleep.

10. Those skilled in the art would also know from the teachings mentioned hereinabove that the DIRC model is useful for predicting the efficacy of compounds that have diverse modes of action. The prior art teaches that the DIRC model is predictive for 5-HT_{1A} antagonists (see Exhibit 3), $GABA_B$ receptor agonists or anticholinergic compounds (see Exhibit 4), "mixed" anticholinergic and calcium antagonist compounds (see Exhibit 5), and tricyclic antidepressants (reuptake inhibitors) (see Exhibit 2). These teachings support the DIRC model's wide range of applicability.

I hereby declare that all statements made herein on my own knowledge are true and that all statements made herein on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

April 18, 2002
Date

Carlos Forray

CURRICULUM VITAE

Carlos Forray, MD

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CURRENT POSITION: *Director*, Cellular Sciences, Automation and Information Technology

APPOINTMENTS:

1999-2000 *Associate Director*, Department of Lead Discovery and Scientific Information Systems, Synaptic Pharmaceutical Corp. Paramus, New Jersey.
1997-1998 *Group Leader*, Lead Discovery, Dept. of Cell Biology, Synaptic Pharmaceutical Corp. Paramus, New Jersey
1993-1996 *Group Leader*, Adrenergic Pharmacology, Dept. Pharmacology, Synaptic Pharmaceutical Corp. Paramus, New Jersey.
1991-1992 *Staff Scientist*, Dept. Pharmacology, Synaptic Pharmaceutical Corp. Paramus, New Jersey.
1988-1991 *Assistant Professor*, Dept. Pharmacology and Toxicology, University of Maryland School of Pharmacy. Baltimore, Maryland.
1985-87 *Associate Professor*, Div. Clinical Research, Instituto Mexicano de Psiquiatría. Secretaría de Salud, México.
1976-83 *Associate Professor*, Dept. Reproductive Biology, Div. of Biological and Health Sciences. Universidad Autónoma Metropolitana, México.

TRAINING:

1982-84 *Senior Research Fellow*, Dept. Pharmacology, Mayo Graduate School of Medicine. Rochester, Minnesota
1973-76 *Fellow*, Dept. Neurobiology, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México.

EDUCATION:

1968–1973 *Medical School*: Facultad de Medicina, Universidad Nacional Autónoma de México

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TEACHING EXPERIENCE:

1989-91	Faculty of the Molecular Biology Program, University of Maryland School of Medicine.
1988-91	University of Maryland School of Pharmacy.
1976-87	Universidad Autónoma Metropolitana, México.

REVIEWER:

1988-	Molecular Pharmacology, Journal of Neurochemistry, Biochemical Pharmacology and Biochemistry, Pharmacology and Behavior, Journal of Medicinal Chemistry, Journal of Pharmacology and Experimental Therapeutics, European Journal of Neuroscience

MEMBERSHIP IN PROFESSIONAL SOCIETIES:

1997-	Society for Biomolecular Screening
1996-	Endocrine Society
1993-	American Urological Association
1986-	Mexican Society for Physiological Sciences
1984-	Mayo Alumni Association
1982-	Society for Neuroscience

GRANT SUPPORT:

1992-94	SBIR, Phase I: Functional Assay Systems for Human Adrenergic Receptors. National Institute for Aging.

AWARDS AND HONORS:

1974	Graduated with honors in Medicine. Universidad Nacional Autónoma de México.

PUBLICATIONS:

A. Original Reports:

49. Chang RS, Chen TB, O'Malley SS, Pettibone DJ, DiSalvo J, Francis B, Bock MG, Freidinger R, Nagarathnam D, Miao SW, Shen Q, Lagu B, Murali Dhar TG, Tyagarajan S, Marzabadi MR, Wong WC, Gluchowski C, Forray C. In vitro studies on L-771,688 (SNAP 6383), a new potent and selective *alpha*1A-adrenoceptor antagonist. *Eur J Pharmacol* 409:301-12, 2000.

48. Bonini JA, Jones KA, Adham N, Forray C, Artymyshyn R, Durkin MM, Smith KE, Tamm JA, Boteju LW, Lakhlani PP, Raddatz R, Yao WJ, Ogozalek KL, Boyle N, Kouranova EV, Quan Y, Vaysse PJ, Wetzel JM, Branchek TA, Gerald C, Borowsky B. Identification and characterization of two G protein-coupled receptors for neuropeptide FF. *J Biol Chem* 275:39324-31, 2000.

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46. Nantermet PG, Barrow JC, Selnick HG, Homnick CF, Freidinger RM, Chang RS, O'Malley SS, Reiss DR, Broten TP, Ransom RW, Pettibone DJ, Olah T, Forray C. Selective *alpha*1a adrenergic receptor antagonists based on 4-aryl-3,4-dihydropyridine-2-ones. *Bioorg Med Chem Lett.* 10:1625-8, 2000.

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42. Wong W.C., Sun W., Cui W., Chen Y., Forray C., Vaysse P.J., Branchek T.A., Gluchowski C. 2-amino-2-oxazolines as subtype selective *alpha*(2) adrenoceptor agonists. *J Med Chem.* 43:1699-704, 2000.

Lagu B, Tian D, Chiu G, Nagarathnam D, Fang J, Shen Q, Forray C, Ransom RW, Chang RS, Vyas KP, Zhang K, Gluchowski C.: Synthesis and evaluation of furo[3,4-d]pyrimidinones as selective *alpha*1a-adrenergic receptor antagonists. *Bioorg. Med. Chem. Lett.* 10:175-8, 2000.

40. Forray C. and Noble S.A.: Subtype selective *alpha*1-adrenoceptor antagonists for the treatment of benign prostatic hyperplasia. *Expert Opinion on Investigational Drugs* 8:2073-2094, 1999.

39. Wong WC, Sun W, Lagu B, Tian D, Marzabadi MR, Zhang F, Nagarathnam D, Miao SW, Wetzel JM, Peng J, Forray C, Chang RS, Chen TB, Ransom R, O'Malley S, Broten TP, Kling P, Vyas KP, Zhang K, Gluchowski C. Design and synthesis of novel *alpha*(1)(a) adrenoceptor-selective antagonists. 4.Structure-activity relationship in the dihydropyrimidine series. *J. Med. Chem.* 42:4804-13, 1999.

38. Lagu B, Tian D, Nagarathnam D, Marzabadi MR, Wong WC, Miao SW, Zhang F, Sun W, Chiu G, Fang J, Forray C, Chang RS, Ransom RW, Chen TB, O'Malley S, Zhang K, Vyas KP, Gluchowski C.: Design and synthesis of novel alpha(1)(a) adrenoceptor-selective antagonists. 3. Approaches to eliminate opioid agonist metabolites by using substituted phenylpiperazine side chains. *J. Med. Chem.* 42:4794-803, 1999

37. Murali Dhar TG, Nagarathnam D, Marzabadi MR, Lagu B, Wong WC, Chiu G, Tyagarajan S, Miao SW, Zhang F, Sun W, Tian D, Shen Q, Zhang J, Wetzel JM, Forray C, Chang RS, Broten TP, Schorn TW, Chen TB, O'Malley S, Ransom R, Schneck K, Bendesky R, Harrell CM, Vyas KP, et al. Design and synthesis of novel *alpha*(1)(a) adrenoceptor-selective antagonists. 2. Approaches to eliminate opioid agonist metabolites via modification of linker and 4-methoxycarbonyl-4-phenylpiperidine moiety. *J. Med. Chem.* 42:4778-93, 1999.

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adrenoceptor-selective dihydropyridine antagonists for the treatment of benign prostatic hyperplasia. *J. Med. Chem.* 41:5320-5333, 1998.

33. Wong, W.C., Chiu, G., Wetzel, J.M., Marzabadi, M.R., Nagarathnam, D., Wang, D., Fang, J., Miao, S.W., Hong, X., Forray, C., Vaysse, P.J., Branchek, T.A., Gluchowski, C., Tang, R., Lepor, H.: Identification of a dihydropyridine as a potent *alpha*1a adrenoceptor-selective antagonist that inhibits phenylephrine-induced contraction of the human prostate. *J. Med. Chem.* 41:2643-2650, 1998.

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29. Wetzel, J.M., Miao, S.W., Forray, C., Borden, L.A., Branchek, T.A., and Gluchowski, C. : Discovery of *alpha* 1a-adrenergic receptor antagonists based on the L-type Ca2+ channel antagonist niguldipine. *J Med Chem* 38:1579- 1581, 199

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27. Lepor, H., Tang, R., Kobayashi, S., Shapiro, E., Forray, C., Wetzel, J.M., and Gluchowski, C.: Localization of the *alpha* 1A-adrenoceptor in the human prostate. *J. Urol.* 154:2096 - 2099, 1995.

26. Wong, W.C., Wang, D., Forray, C., Vaysse, P.J.-J., Branchek, T.A., and Gluchowski, C.: A convenient synthesis of 2-amino-2-oxazolines and their pharmacological evaluation at cloned human adrenergic receptors. *Bioorg. Med. Chem. Let.* 4: 2317-2322, 1994.

25. Laz, T.M., Forray, C., Smith, K.E., Bard, J.A., Vaysse, P.J., Branchek, T.A., and Weinshank, R.L. : The rat homologue of the bovine *alpha* 1c-adrenergic receptor shows the pharmacological properties of the classical *alpha* 1A subtype. *Mol. Pharmacol.* 46: 414 - 422, 1994.

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23. Michel, M.C., J. Kerker, T.A. Branchek, and Forray.C. : Selective irreversible of cloroethylclonidine at 1- and 2-adrenoceptor subtypes. *Mol. Pharmacol.*, 44: 1165-1170, 1993.

22. Kiefer-Day J.S., Abdallah E.A.M., Forray, C., Lee N.H., Kim O.N., and El-Fakahany E.E.: Effects of tacrine on brain muscarinic-receptor-mediated second-messenger signals. *Pharmacol.* 47: 84-90, 1993.

21. Mohamed A.S., Forray, C., Aly M.H. and El-Fakahany E.E.: Lack of intrinsic activity and significant subtype selectivity of SR 95639A at muscarinic receptors. *Eur. J. Pharmacol.* 227: 181-187, 1992.

20. Hu J., Wang S.-Z., Forray, C. and El-Fakahany E.E.: Complex allosteric modulation of cardiac muscarinic receptors by protamine: potential model for putative endogenous ligands. *Mol. Pharmacol.* 42: 311 321, 1992.

19. Arroyo C.M. and Forray, C.: Activation of cyclic GMP formation in mouse neuroblastoma cells by a labile nitroxyl radical. An electron paramagnetic resonance/spin trapping study. *Eur. J. Pharmacol.* 208: 157-161, 1991.

18. Fernando J.C.R., Abdallah E.A.M., Evinger M., Forray, C. and El-Fakahany E.E.: The presence of an M_4 subtype muscarinic receptor in the bovine adrenal medulla revealed by mRNA and receptor binding analyses. *Eur. J. Pharmacol.* 207: 297-303, 1991.

17. Wang S.-Z., Hu J., Long R. M., Pou W.S., Forray, C., and El-Fakahany E. E.: Agonist-induced down-regulation of m1 muscarinic receptors and reduction of their mRNA level in a transfected cell line. *FEBS Letters.* 276: 185-188, 1991.

16. Arroyo C.M., Forray, C., El-Fakahany E.E., and Rosen G.M.: Receptor-mediated generation of an EDRF-like intermediate in a neuronal clone detected by spin trapping techniques. *Biochem. Biophys. Res. Commun.* 170: 1177-1183, 1990.

15. Forray, C., and El-Fakahany E.E.: On the involvement of multiple muscarinic receptor subtypes in the activation of phosphoinositide metabolism in rat cerebral cortex. *Mol. Pharmacol.* 37: 893-902, 1990.

14. Abdallah E.A.M., Forray, C., and El-Fakahany E.E.: Relationship between the partial inhibition of muscarinic receptor-mediated phosphoinositide hydrolysis by phorbol esters and tetrodotoxin in rat cerebral cortex. *Mol. Brain Res.* 8: 1-7, 1990.

13. Surichamorn W., Forray, C., and El-Fakahany E.E.: The role of intracellular Ca^{2+} mobilization in muscarinic and histamine receptor-mediated activation of guanylate cyclase in N1E-115 neuroblastoma Cells. Evidence against the arachidonic acid release hypothesis. *Mol. Pharmacol.* 37: 860-869, 1990.

12. Lee N.H., Fryer A.D., Forray C., and El-Fakahany E.E.: Different mechanisms of antagonism by Methoctramine of two neuronal muscarinic receptor-mediated second messenger responses. *J. Pharmacol. Exptl. Ther.* 251: 992-999, 1989.

11. McKinney M., Anderson D.J., Forray C., and El-Fakahany E.E.: Characterization of the striatal M2 muscarinic receptor mediating inhibition of cyclic AMP using selective antagonists: A comparison with the brainstem M2 receptor. *J. Pharmacol. Exptl. Ther.* 250: 565-572, 1989.

10. Lee N.H., Forray C., El-Fakahany E.E.: Methoctramine, a cardioselective muscarinic antagonist, stimulates phosphoinositide hydrolysis in rat cerebral cortex. *Eur. J. Pharmacol.* 167: 295-298, 1989.

9. Surichamorn, W., Amrhein C.L., Forray C. and El-Fakahany E.E.: Inhibition of cyclic AMP formation in N1E-115 neuroblastoma cells is mediated by a non-cardiac M_2 muscarinic receptor subtype. *Brain Research* 493: 320-325, 1989.

8. Anton-Tay F., Forray C., and B.G. Ortega-Corona: Subneuronal fate of intracerebroventricular injected [^{3}H]-melatonin. *J. Pineal Res.* 5: 125-134, 1988.

7. Heinze G., Ortega G., Benitez G., Forray C., Huerto L., Galvan G., de la Fuente J.R., and Salas C.: Density of putative dopaminergic receptors in lymphocytes of patients with paranoid schizophrenia. Preliminary results. *Salud Mental* 11: 1-6, 1988.

6. Richelson E., Stenstrom S., Forray C., Enloe L. and Pfenning M.: Effects of chronic exposure to ethanol on the Prostaglandin E_1 receptor mediated response and binding in a murine neuroblastoma clone (N1E-115). *J. Pharmacol. Exptl. Ther.* 239: 687-692, 1986

5. Abraham, R.T., McKinney, M.M., Forray, C., Shipley, G.D., and Handwerger, B.S.: Stimulation of arachidonic acid release and eicosanoid biosynthesis in an interleukin 2-dependent T cell line. *J Immunopharmacol* 8:165 - 204, 1986.

4. Snider R.M., Forray C., Pfenning M. and Richelson E.: Neurotensin stimulates inositol phospholipid metabolism and calcium mobilization in murine neuroblastoma clone N1E-115. *J. Neurochemistry* 47: 1214-1218, 1986.

3. Forray C., and Richelson E.: Glucocorticoids potentiate the prostaglandin E_1-mediated cyclic AMP formation by a cultured murine neuroblastoma clone. *J. Neurochemistry* 45: 79-85, 1985.

2. Snider R.M., McKinney M., Forray C., and Richelson E.: Neurotransmitter receptors mediate cyclic GMP formation by involvement of arachidonic acid release and lipooxygenase. *Proc. Natl. Acad. Sci. USA* 81:3905-3909, 1984.

1. Mena F., Martínez-Escalera G., Clapp C., Aguayo D., Forray C. and Grosvenor C.E.: Analysis of the mechanisms depletion-transformation and repletion of prolactin by the lactating rat pituitary; *in vitro* studies. *Endocrinology* 111:1086-1091, 1982.

B. Paper Presentations and Abstracts:

66. Wong, W., Zhang, J., Lagu, B., Marzabadi, M., Dhar, T., Wetzel, J., Miao, S., Tang, J., Zhang, F., Sun, W., Tian, D., Forray, C., Gluchowski, C., Patane, M., Chang, R., Broten, T., Schorn, T., Chen, T., O'Malley, S., Ransom, R., Schneck, K., Bendesky, R., Harrell, C., Kling, P., and Pettibone, D.: *Alpha*-1a adrenoceptor selective antagonists as novel agents for treating benign prostatic hyperplasia. *National Meeting of the ACS*, 1999.

65. Schorn, T., Broten, T., Siegl, P., Bock, M., Payne, L., Patane, M., Lagu, B., Nagarathnam, D., Wong, W., Marzabadi, M.R., Muarli Dhar, T., Forray, C., and Gluchowski, C.: The *alpha* -1d adrenergic receptor (AR) subtype contributes to the phenylephrine (PE)- induced increase in canine prostatic intraurethral pressure (IUP). *FASEB J.* 13:A , 1999.

64. Chen, T., O'Malley, S., Nagarathnam, D., Miao, S., Wong, W., Lagu, B., Marzabadi, M., Forray, C., Gluchowski, C., Pettibone, D., and Chang, R.: In vitro pharmacology of SNAP 6991: an *alpha* 1A-adrenergic selective antagonist. *FASEB J.* 13:A150.3, 1999.

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8. "Heterogeneity of Galanin Receptors: Expression Cloning and Pharmacological Characterization." IBC Conference on G-protein Coupled Receptors. San Diego, USA 1997.
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EFFECTS OF SOME ANTIDEPRESSANTS ON THE VOLUME-INDUCED REFLEX CONTRACTIONS OF THE RAT URINARY BLADDER: LACK OF CORRELATION WITH MUSCARINIC RECEPTORS AFFINITY

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SUMMARY

It has been suggested that tricyclic antidepressants such as imipramine, might exert their anti-enuretic action by a blockade of muscarinic receptors in the detrusor muscle of the urinary bladder. We have therefore investigated the effects of two tricyclic (imipramine and nortriptyline) and three atypical (citalopram, amineptine and mianserin) antidepressants on the micturition reflex and muscarinic receptors in rats. The micturition reflex pathway was monitored indirectly by recording the rhythmic intravesical pressure waves which occurred when the bladder was distended and maintained under constant saline-volume. The activity of the antidepressants was correlated to their potencies as antagonists of [^{3}H]QNB binding to rat brain (mainly M1 receptors) and bladder (mainly M2 receptors) membranes, as well as antagonists of carbachol-induced contractions of rat bladder strips. Only imipramine and citalopram dose dependently inhibited the voiding contractions, whereas nortriptyline, imipramine and mianserin (in order of potency) were active both in binding studies and as competitive antagonists of carbachol-induced bladder contractions, but were inactive in inhibiting the micturition reflex. The present data seem to suggest that affinities for muscarinic receptors are unrelated to the inhibition of micturition reflex.

KEY WORDS: antidepressants, micturition reflex, muscarinic receptors.

INTRODUCTION

The detrusor and the bladder outlet are a coordinated unit permitting storage and expulsion of urine. Urine storage and timely expulsion of bladder content are produced through the coordinated activation of a series of reflexes involving

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cholinergic, sympathetic and, possibly, purinergic, serotonergic and peptidergic innervation [1, 2 and references therein]. In both animals and humans micturition is initiated and maintained through the activation of a supraspinal vesicovesical micturition reflex pathway which can be monitored indirectly by recording the rhythmic, large amplitude intravesical pressure waves which occur when the bladder is distended and maintained under constant volume conditions [3–5].

Tricyclic antidepressants, particularly imipramine, have come to be accepted for the treatment of enuresis and a number of other micturition disorders [6–8]. Despite the conflicting reports about their mechanism of action, most of the evidence suggests that their activity might be related to a preferential blockade of cholinergic muscarinic receptors in the detrusor muscle [9]. However, most of the evidence on which this assumption is based has been obtained by *in vitro* studies on whole bladder or bladder strips of different animal species [10–12] as well as on muscarinic receptor populations in homogenized tissue preparations [9, 12].

We have therefore investigated *in vivo*, the effects of some antidepressants belonging to different chemical structures on the rat micturition reflex. The relationship between *in vivo* activities and *in vitro* affinities with rat brain (mainly M1) and bladder (mainly M2) muscarinic receptors [13], as well as the inhibitory effects against the carbachol-induced contractions of rat bladder strips, were also investigated. Imipramine and nortriptyline were chosen as tricyclic antidepressants with proven affinity for muscarinic receptors and as drugs utilized in the therapy of enuresis or depression associated with incontinence. Citalopram, amineptine and mianserin, second generation antidepressants, were studied to assess if these new drugs are endowed with an inhibitory effect on the volume-induced bladder contractions and if this effect could be related to an anticholinergic activity.

MATERIALS AND METHODS

Three batches of (−)[^{3}H]quinuclydinyl benzilate, [^{3}H]QNB, with specific activity of 31·9, 43·5 and 45·7 Ci/mmol respectively were used (New England Nuclear). Antidepressants were obtained from commercial sources. Male and female Sprague–Dawley rats [Crl:CD° (SD) BR] from Charles River Italia, weighing 200–250 g, were housed five/cage, with food and water *ad libitum* and kept in an animal room at constant temperature of 22°C with a 12 h alternating light–dark cycle.

Rat urinary bladder preparations

The *in vivo* studies were carried out according to the method reported originally by Dray [14] with some modifications. The urinary bladder of anaesthetized rats (urethane 1·5 g/kg i.m.) was catheterized via the urethra by use of PE 50 polyethylene tubing filled with saline.

The catheter was tied in place with a ligature around the external urethral orifice and intravesical pressure was monitored by a conventional pressure transducer (Gould-Statham P23ID) and displayed continuously on a chart recorder (Battaglia-Rangoni).

The bladder was filled via the recording catheter by incremental volumes of warmed (37°C) saline until bladder contractions occurred (usually 0·8–1 ml) as a result of central activity. Volume-induced contractions were then recorded and occurred rhythmically and reproducibly for 2–3 h in individual animals. Drug activity was assessed in each animal against the background frequency of bladder contractions, for a 15-min time period following intravenous administration of different doses. In some experiments, rats treated with the higher dose utilized (4 mg/kg) were also prepared in order to study the cardiovascular effects of the antidepressants, inserting a catheter into the carotid artery for the measurement of blood pressure by a pressure transducer and a polygraph.

The *in vitro* inhibition of carbachol-induced contractions was studied on rat detrusor strip preparations. The rats were sacrificed by decapitation and the bladder was taken out and immediately placed in Krebs solution (for composition, see below). The detrusor muscle tissue (bladder dome) was cut in a semicircular direction and further dissected into strip preparations measuring approximately 2 × 20 mm. The strip preparations were transferred to 10 ml organ baths containing Krebs solution of the following composition (mM): NaCl 118, KCl 4·6, $CaCl_2$ 1·5, $MgSO_4 \cdot 7H_2O$ 1·11, KH_2PO_4 1·5, $NaHCO_3$ 25 and glucose 11. The Krebs solution was continuously bubbled with a mixture of CO_2 (5%) and O_2 (95%) and the tension of the preparations was adjusted during an equilibration period of approximately 1 h to a final level of 1 g.

Carbachol was added cumulatively to the bath and the concentration was increased only after the response to the previous addition had attained a steady level. Different concentrations of testing drugs were then added to the bath and after 30 min of incubation, a second cumulative dose–response curve of carbachol was performed.

[^{3}H]QNB binding studies

Cholinergic muscarinic binding sites were assayed in rat brain (minus cerebellum) membranes according to the method described by Yamamura and Snyder [15] with minor modifications. For these studies, male rats were decapitated and the brain rapidly removed and homogenized in 10 volumes of 0·32 M sucrose using a Polytron homogenizer. The homogenates were centrifuged (1000 *g*) for 10 min and the supernatants again homogenized and used immediately. Aliquots of 30 μl were incubated for 60 min at 25°C in 1 ml of 0·05 M Na-K buffer (pH 7·4) containing 10 μl of [^{3}H]QNB 0·05 nM (final concentration), 10 μl of unlabelled drug (dissolved in ethanol) or 10 μl of ethanol. Incubation was done in 1 ml polyethylene tubes, and was terminated by rapid filtration through Whatman GF/B filters using semiautomatic equipment (Skatron). Filters were washed three times with cold phosphate buffer (4 ml total). Radioactivity was quantified by liquid scintillation spectrometry (LKB 1217 Rackbeta liquid scintillation counter), in vials with 4 ml of filter-count (Packard). Specific muscarinic binding was defined as the difference between the total binding and that remaining in the presence of atropine 10 μM. The affinity for bladder muscarinic receptors was assayed according to Nilvebrant *et al.* [16]. Male rats were killed by fracture of the cervical spine; the bladders were rapidly removed, cleaned, weighed and, if necessary, frozen at

−80°C. The bladders were homogenized in 10 volumes of an ice-cold 0·05 M Na-K buffer (pH 7·4) containing 10^{-3} M phenylmethylsulphonyl fluoride (PMSF, a protease inhibitor), using Polytron equipment. The homogenates were diluted with the ice-cold phosphate/PMSF buffer to 375 volumes of the original wet weight and filtered through a double layer of gauze. Aliquots of 1 ml of homogenates were incubated for 3 h at 25°C with 10 μl [^{3}H]QNB (1 nM final concentration), 10 μl of unlabelled drug (dissolved in ethanol) or 10 μl of ethanol. For filtration and determination of specific binding, see above.

Statistical analysis

Owing to the high level of variability observed in the inhibition of frequency of the bladder voiding contractions, the all-or-none criterion was introduced to assess drug effects by considering positive effect when the inhibition was ⩾50%. Quantal dose–response curves were evaluated by the method of Bliss [17], computerized on Wang PC according to the indications reported by Rosiello *et al.* [18].

Dose–response curves (binding studies and carbachol-induced contractions) were always analysed by simultaneous non-linear curve fitting of the logistic equation: $y=(a-d)/[1+(x/c)^b]+d$ according to the method reported by De Lean *et al.* [19]. In the equation y is the response; x the arithmetic dose; a the response when $x=0$; d the response for 'infinite' dose; c is the IC_{50}, i.e. the concentration resulting in a response halfway between a and d; and b is a slope factor that determines the steepness of the curve. Curve fittings were performed on IBM PC-AT utilizing the ALLFIT program (from NIH). In the binding studies, the IC_{50} values (and the corresponding 95% confidence limits evaluated by ALLFIT), were converted in the apparent inhibition constants (K_i) using the equation: $K_i=IC_{50}/(1+[L]/K_d)$ [20]. In the carbachol cumulative dose–response curves, a value was always constant and = 0. Schild-plot parameters (pA2 and pD′2) were evaluated by linear regression analysis using a LOTUS spreadsheet according to Tallarida and Murray [21].

RESULTS

Intravenous administration of different doses of imipramine and citalopram inhibited the high-amplitude and low-frequency rhythmic intravesical pressure waves which occurred when the bladder was distended and maintained under constant volume conditions. These effects occurred rapidly and were also characterized by a slight non-dose-related decrease in the amplitude of the contractions, as shown in Fig. 1. The number of animals showing more than 50% of inhibition in the frequency of voiding contractions increased dose dependently after these two drugs (Table I), and allow us to calculate an ED_{50} value of 1·08 (0·63–1·86; 95% CL) and 2·23 (1·42–3·49; 95% CL) mg/kg for citalopram and imipramine respectively. The administration of nortriptyline and mianserin induced in some animals a marked inhibition of the contractions, but the effects were not dose-dependent. Moreover, in some animals these two antidepressants induced also a marked (> 50%) increase in the frequency (3/15 at 1 mg/kg for nortriptyline and 2/5 at 0·5 mg/kg for mianserin). Amineptine was virtually devoid of effects in

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this test. In order to exclude whether the inhibition of bladder motility was influenced by cardiovascular changes produced by i.v. administration of the antidepressants, data about changes in blood pressure were obtained after the administration of the higher dose utilized (4 mg/kg).

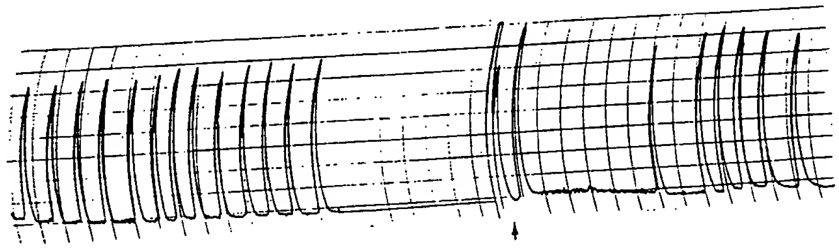

Fig. 1. Typical tracing showing the effects of i.v. administration of antidepressants (imipramine 2 mg/kg) on volume-induced contractions of rat bladder. Arrow indicates the drug injection. Each square represents 1 min (horizontal) or 5 mmHg (vertical).

Table I

Inhibitory effects of different i.v. doses of antidepressants on frequency of volume-induced contractions of rat urinary bladder

Drugs	*Doses (mg/kg)* 0·25	0·50	1·00	2·00	4·00
Imipramine	n.t.	0/4	2/7	2/7	8/10
Nortriptyline	0/5	4/13	4/15	4/10	1/14
Citalopram	0/5	2/8	5/7	4/7	6/7
Amineptine	0/8	2/8	2/8	0/9	1/7
Mianserin	0/5	3/5	1/5	2/7	2/9

Data represent the number of animals showing more than 50% of inhibition of voiding contractions/number of animals tested.

n.t., not tested.

Citalopram and amineptine showed no consistent effects on systolic and diastolic blood pressure 2 and 15 min after administration. Nortriptyline decreased mainly diastolic blood pressure (−47%) immediately after drug injection (2 min), but its effect disappeared 15 min after the administration. Mianserin and imipramine induced a consistent reduction in blood pressure (−69 and −54% respectively of diastolic blood pressure, 2 min after administration) that was still present 15 min after injection (−29 and −28% respectively). The inhibition of the voiding

contractions appeared, therefore, unrelated to the cardiovascular effects of the tested antidepressants.

To test whether the inhibition of bladder voiding contractions exerted by antidepressants could be related to an antagonism against the muscarinic receptors, binding studies on [^{3}H]QNB displacement from rat brain and bladder membranes were performed, together with functional studies in which the compounds were tested on carbachol-induced contractions of rat bladder strips. The affinity data obtained from binding competition curves are summarized in Table II. In our experimental conditions, K_d values of the tritiated ligand were 0·104 and 0·16 nM for brain and bladder membranes respectively. Nortriptyline and imipramine were more active on M1 than on M2 receptors (nortriptyline being more active than imipramine), showing K_i values in the nanomolar range. Mianserin was equally potent on both types of receptors, and its K_i was close to the micromolar concentration. Citalopram behaved as mianserin but was less active with a K_i value of about 10 μM. Amineptine was always inactive. The slopes of the curves were close to unity, suggesting that the inhibition curves are compatible with a one-binding site model.

Table II

Inhibition of [^{3}H]QNB binding in rat brain (muscarinic M1 receptors) and urinary bladder (muscarinic M2 receptors) by antidepressants

Drugs	*Brain* K_i (nM)	*Slope*	*Bladder* K_i (nM)	*Slope*
Imipramine	317 (284–355)	1·01	1150 (811–1635)	0·90
Nortiptyline	208 (156–278)	0·94	896 (732–1100)	1·05
Citalopram	9089 (4069–20 299)	0·92	12 503 (9618–16 253)	0·93
Amineptine	⩾ 10 000	—	⩾ 10 000	—
Mianserin	2095 (1862–2648)	1·01	1416 (1010–1987)	0·90
Atropine	4 (3–5)	0·90	6 (5–8)	1·02

Data represent the apparent inhibition constants (K_i), the 95% confidence limits (in parentheses) and the slopes of the curves, evaluated as reported in Methods.

Imipramine, nortriptyline and mianserin were also the most potent compounds in inhibiting the carbachol-induced contractions of rat bladder strips (Table III). In the presence of the lower concentrations of the compounds, there was an almost

parallel dose-dependent shift to higher agonist concentration of the cumulative dose–response curve, similar to that exerted by atropine (Figs 2 and 3 as example of antidepressant antagonism). At the higher concentrations tested, all three compounds depressed the carbachol maximum response, indicating that they are

Table III
Parameters of Schild plots of antidepressant antagonism of carbachol-induced contractions in rat bladder strips

Drugs	K_i (nM)	$pA2 \pm SE$	$pD'2 \pm SE$
Imipramine	269	6·57 ± 0·13	5·48 ± 0·10
Nortriptyline	213	6·67 ± 0·12	5·09 ± 0·04
Citalopram	—	—	4·92 ± 0·14
Amineptine	—	—	⩽4·50
Mianserin	371	6·43 ± 0·12	5·04 ± 0·04
Atropine	0·63	9·20 ± 0·13	—

K_i represents the equilibrium dissociation constant and pA2 the $-\log(K_i)$. pD'2 values represent the concentration of antagonist that depressed by 50% the maximum of the carbachol-induced contraction.
—, not present.

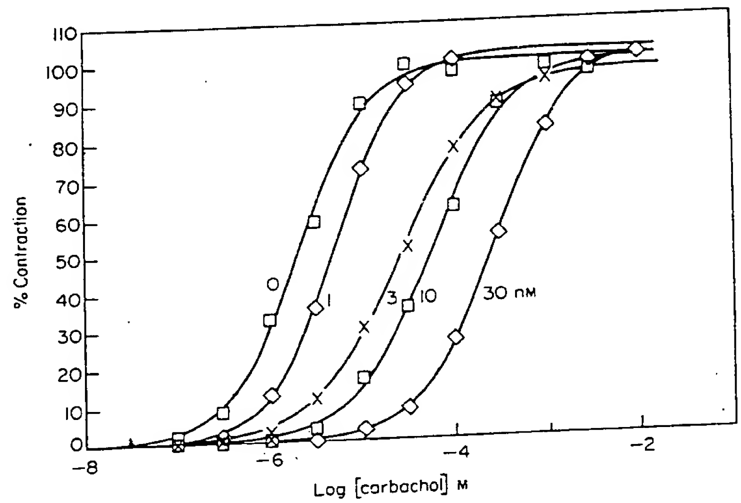

Fig. 2. Dose–response curves of the contractile effect of carbachol in rat bladder (as per cent of the maximum effect) and in the presence of increasing concentrations of atropine. Each point represents the mean of 2–3 experiments. Curves were fitted by the logistic equation (see Methods). Drug concentrations are indicated on the curves.

not pure competitive antagonists. Citalopram (Fig. 4) showed a non-competitive antagonism. Although the carbachol maximum contraction was significantly decreased by increasing doses of the compound, no changes in the ED_{50} of the agonist were observed at concentrations up to 15·6 μM. Amineptine was completely inactive up to a concentration of 3×10^{-5} M.

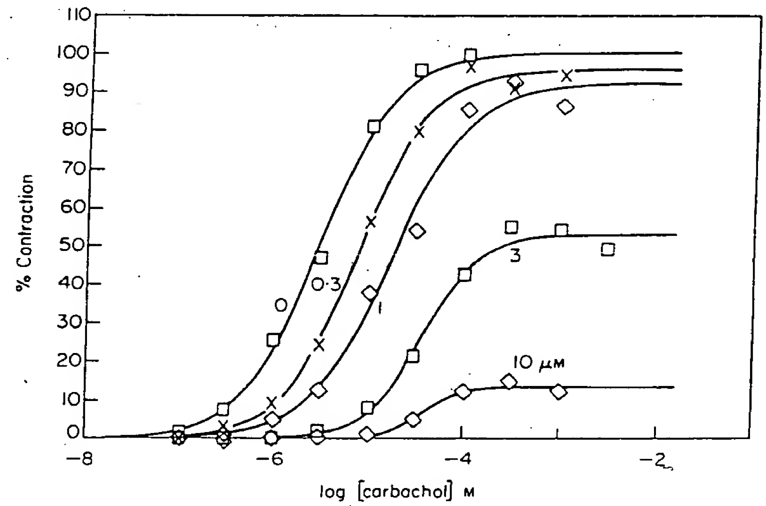

Fig. 3. Dose–response curves of the contractile effect of carbachol in rat bladder and in the presence of increasing concentrations of imipramine. Other details as indicated in Fig. 2.

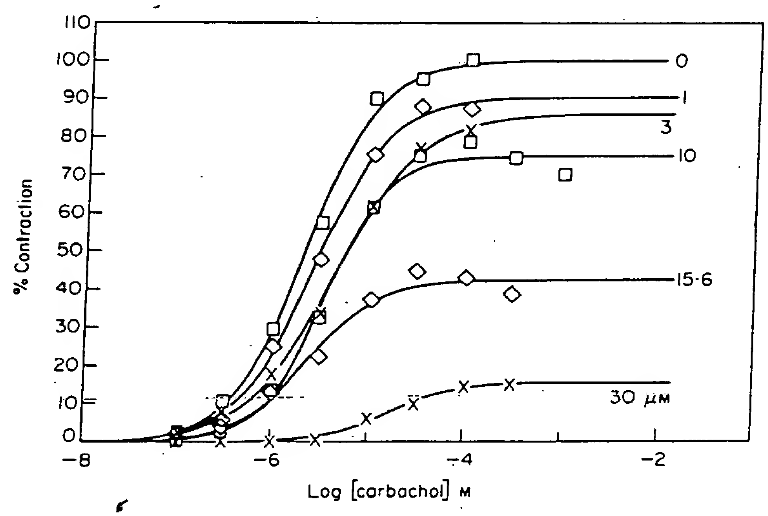

Fig. 4: Dose–response curves of the contractile effect of carbachol in rat bladder and in the presence of increasing concentrations of citalopram. Other details as indicated in Fig. 2.

DISCUSSION

This investigation was prompted by the hypothesis that some tricyclic antidepressants owe their anti-enuretic action to a preferential blockade of cholinergic muscarinic receptors in the detrusor muscle of urinary bladder, as reviewed by Blackwell and Currah [6]. We have therefore examined the effects of two tricyclic (imipramine and nortriptyline) and three atypical (citalopram, amineptine and mianserin) antidepressants on muscarinic receptors binding sites of rat brain and bladder labelled by [^{3}H]QNB, on carbachol-induced contractions of rat bladder strips and on volume-induced micturition reflexes of rat urinary bladder.

Nortriptyline, imipramine, mianserin and citalopram (in order of potency) displaced [^{3}H]QNB from rat brain membranes, whereas amineptine was inactive. These results are in agreement with previously reported data [22–24]. Imipramine and nortriptyline were significantly more active on brain M1 receptors than on bladder M2 binding sites, whereas mianserin and citalopram, in our experimental conditions, do not discriminate between M1 and M2 muscarinic receptors.

It has been reported that tricyclic antidepressants preferentially antagonize M2 sites [24]. This conclusion is based on the finding that in rat brain the antidepressants are much more potent antagonists of carbachol-induced inhibition of adenylate cyclase than of carbachol stimulation of inositol phosphates accumulation. In rat brain, phosphatidyl inositol accumulation is associated with muscarinic receptors of the M1 subtype, whereas adenylate cyclase is regulated by M2 sites [24]. However, in the bladder [25, 26] both second messenger systems seem to be associated with M2 receptors sites, that therefore appear different from the central ones. This difference in the nature of bladder M2 receptors could justify our findings. Moreover, it has also been reported [27] that imipramine shows better affinity for cortex than for guinea-pig bladder muscarinic receptors.

Nortriptyline, imipramine and mianserin, at the lower concentrations tested, showed a competitive antagonism against carbachol-induced rat bladder contractions, whereas citalopram was a non-competitive antagonist and amineptine was inactive. At higher doses, all the competitive antagonists significantly depressed the maximum response of carbachol. These findings might indicate that other mechanisms of action are implicated in the inhibition of the *in vitro* muscarinic-induced contractions, such as an inhibition of the participation of Ca^{2+} in the excitation–contraction coupling process as reported for imipramine [10, 28].

Among the antidepressants tested, citalopram and imipramine only depressed the frequency of micturition reflex in a dose-dependent way. Nortriptyline and mianserin showed a dual effect (inhibitory and excitatory) and amineptine was practically inactive. Taken together, these results seem to indicate a lack of correlation between the activities on muscarinic receptors and the inhibition of micturition reflex.

The observed effects on bladder contractions could be due to other mechanisms not involving acetylcholine, such as prevention of monoamines uptake. The antidepressants used are all reuptake inhibitors but with varying selectivities for noradrenaline (norepinephrine), serotonin and dopamine. Nortriptyline and mianserin are highly noradrenaline-selective, imipramine is noradrenaline- and

serotonin-selective, citalopram is serotonin-specific and amineptine is dopamine-specific [29, 30].

Distension of the urinary bladder in urethane-anaesthetized rats activates a supraspinal micturition reflex whose efferent limb travels through the parasympathetic pelvic nerves. Bladder distension also activates an inhibitory sympathetic reflex, travelling through the hypogastric nerves, which counteracts the excitatory reflex [4, 31–33]. Therefore, the parasympathetic and the sympathetic division of the autonomic nervous system play opposite róles on bladder responsiveness to distension.

When tested in experimental conditions involving activation of both excitatory and inhibitory reflexes to the bladder, clonidine produced either inhibitory and/or excitatory effects on bladder motility [4]. These observations could explain the opposite effects exerted by mianserin and nortriptyline which are mainly noradrenaline uptake inhibitors.

Some evidence for participation of spinal serotonergic and adrenergic descending pathways in regulation of bladder tone in rats was recently reported by Durant and Yaksh [34]. Moreover, Maggi *et al.* [35] indicated that functional integrity of serotonergic transmission is required for imipramine to exert an acute inhibition on the threshold of the spinal vesicovesical reflex, since the effect of the drug was almost abolished by *p*-chlorophenylalanine, that depletes the serotonin containing nerves. The possible implication of serotonin as inhibitory transmitter of certain micturition pathways is also suggested by our results. In fact, citalopram (a specific serotonin uptake inhibitor) and imipramine (a mixed inhibitor) were the only active compounds in inhibiting the frequency of voiding contractions.

In considering other possible mechanisms of action, Dray [14] observed that i.c.v. administration of desipramine suppressed the volume-induced contractions of the rat bladder and suggested a centrally mediated action of antidepressants.

Several published papers provide convincing evidence that endogenous opioid peptides mediate a tonic inhibition of detrusor reflex pathways [2, 36–38] and that acute administration of antidepressants induces an increase in central endogenous opioid levels in the rat [39, 40]. In studying the effects of citalopram, amineptine, imipramine and nortriptyline on stress-induced analgesia in rats (an endogenous opioid peptides-dependent effect) Testa *et al.* [41] reported the following order of potency in increasing the analgesia induced by inescapable footshock: citalopram $\gg$ imipramine $>$ nortriptyline $\gg$ amineptine (which was almost inactive).

Therefore, additional investigations are now in progress to test if the inhibitory effects of antidepressants on spontaneous volume-induced bladder contractions might be related to the modulatory effects of these drugs on the endogenous opioid peptides involved in the micturition reflex pathways.

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(54) Title: USE OF 5-HT_{1A} RECEPTOR ANTAGONISTS FOR THE TREATMENT OF URINARY INCONTINENCE

(57) Abstract

Compounds which: (a) bind to a 5-HT_{1A} receptor with an affinity of at least 10^{-7}M, (b) bind to a 5-HT_{1A} receptor with an affinity at least 50-fold stronger than the affinity with which the compound binds to an α_1-adrenergic receptor, and (c) exhibit 5-HT_{1A} receptor antagonist activity on both pre-synaptic and post-synaptic 5-HT_{1A} receptors, and their stereoisomers, hydrates, solvates and pharmaceutically acceptable salts are useful for the treatment of lower urinary tract disorders in mammals.

Applicants: Douglas A. Craig
U.S. Serial No.: 09/450,880
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Exhibit 3

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TITLE

Use of 5-HT_{1A} Receptor Antagonists for the Treatment of Urinary Incontinence

DESCRIPTION

The invention relates to compositions and methods for treating certain disorders of the lower urinary tract in mammals, including humans, using serotonin 5-HT_{1A} receptor antagonist compounds which exert their inhibitory effects via both pre-synaptic (somatodendritic) and post-synaptic antagonism.

In mammals, micturition is a complex process which requires the integrated actions of the bladder, its internal and external sphincters, the musculature of the pelvic floor, and neurological control over these muscles at three levels (in the bladder wall or sphincter itself, in the autonomic centres of the spinal cord, and in the central nervous system at the level of the pontine micturition centre in the brainstem (pons) under the control of the cerebral cortex). Micturition results from contraction of the detrusor muscle, which consists of interlacing smooth muscle fibres under parasympathetic autonomic control from the sacral spinal cord. A simple voiding reflex is formed by sensory nerves for pain, temperature, and distension that run from the bladder to the sacral cord. However, sensory tracts from the bladder also reach the pontine micturition centre, resulting in the generation of nerve impulses that normally suppress the sacral spinal reflex arc controlling bladder emptying. As a result, normal micturition is initiated by voluntary suppression of cortical inhibition of the reflex arc and by relaxation of the muscles of the pelvic floor and the external sphincter. Finally, the detrusor muscle contracts and voiding occurs.

Functional abnormalities of the lower urinary tract, e.g., dysuria, incontinence, and enuresis, are common in the general population. Dysuria includes urinary frequency, nocturia, and urgency, and may be caused by cystitis, prostatitis or benign prostatic hypertrophy (which affects about 70% of elderly males), or by neurological disorders. Incontinence syndromes include stress incontinence, urgency incontinence, and overflow incontinence. Enuresis refers to the involuntary passage of urine at night or during sleep.

Prior to the present invention, treatment of neuromuscular disorders or the lower urinary tract has involved administration of compounds which act directly on the bladder muscles, such as flavoxate, a spasmolytic drug also active on the pontine micturition centre, or anticholinergic compounds such as oxybutynin. The use of α_1-adrenergic receptor antagonists for the treatment of benign prostatic hypertrophy is

also common. However, treatments that involve direct inhibition of the pelvic musculature (including the detrusor muscle) may have unwanted side effects such as incomplete voiding or accommodation reflex paralysis, tachycardia and dry mouth. Thus, it would be preferable to utilize compounds which act via the peripheral or central nervous system, for example to affect the sacral spinal reflex arc and/or the inhibition pathways of the pontine micturition centre in a manner that restores normal functioning of the micturition mechanism.

Lecci et al (*J. Pharmacol. Exp. Therapeutics*, 262, 181, 1992) describe the effects of the 5-HT_{1A} receptor ligands 8-hydroxy-2-(di-N-propylamino)-tetralin (8-OH-DPAT) and 1-(2-methoxyphenyl)-4-[4-(2-phthalimido)-butyl]-piperazine (NAN-190) on micturition reflexes in the anaesthetized rat. 8-OH-DPAT (an agonist) stimulated the supraspinal micturition reflex originating from the pontine micturition centre, while NAN-190 inhibited the supraspinal micturition reflex. The authors concluded that spinal and supraspinal 5-HT_{1A} receptors modulate the supraspinal micturition reflex in this system. The present inventors, however, have found that the efficacy of NAN-190 and other 5-HT_{1A} receptor ligands in inhibiting the supraspinal micturition reflex is directly correlated to their relative binding affinities for the α_1-adrenergic receptor, rather than to their affinities, if any, for the 5-HT_{1A} receptor which called into question the relevance of the effects correlated to 5-HT_{1A} receptor activity for lower urinary tract disorders. Finally, as discussed below, NAN-190 is considered a partial 5-HT_{1A} receptor agonist rather than a complete or "true" antagonist. Therefore, prior to the present invention, the use of true 5-HT_{1A} receptor antagonists to treat urinary tract disorders was unknown.

At least two functionally distinct types of the 5-HT_{1A} receptor have been identified, and these are designated "pre-synaptic" (or somatodendritic) and "post-synaptic". Pre-synaptic receptors are present on 5-HT-producing neurons and are involved in autoregulation of 5-HT release; their activation causes physiological changes including hyperphagia, hypothermia (in the mouse), bradycardia and hypotension. Post-synaptic receptors are widely distributed throughout the mammalian brain and are coupled to potassium channels and adenylate cyclase; their activation leads to "5-HT behavioural syndrome", hypothermia (in the rat), and elevation of plasma corticotropin levels. Beyond the differences in their anatomical distribution and functioning, pre-synaptic and post-synaptic receptors can be distinguished by the differential activity profiles of different 5-HT_{1A} receptor ligands. For example, full agonists such as 8-OH-DPAT and 5-carboxytryptamine have agonist activity on both pre-synaptic and post-synaptic receptors. By contrast, partial agonists such as buspirone, ipsapirone, spiroxantine,

urapidil, NAN-190 and BMY 7378 have agonist activity on pre-synaptic receptors and antagonist activity on post-synaptic receptors. Finally, some compounds exhibit antagonistic activity on both pre-synaptic and post-synaptic receptors. These last compounds, true antagonists of the serotoninergic 5-HT_{1A} receptor, are useful in the invention.

The invention provides the use of a compound which

(a) binds to a 5-HT_{1A} receptor with an affinity of at least 10^{-7} M,

(b) binds to a 5-HT_{1A} receptor with an affinity at least 50 times greater than the affinity with which the compound binds to an α_1-adrenergic receptor, and

(c) exhibits 5-HT_{1A} receptor antagonist activity on both pre-synaptic and post-synaptic 5-HT_{1A} receptors,

or of a stereoisomer, hydrate, solvate or pharmaceutically acceptable salt of such a compound, for the preparation of a medicament for the treatment of neuromuscular disorders of the lower urinary tract in mammals.

Compounds useful in the practice of the invention preferably bind to 5-HT_{1A} receptors with an affinity (K_i) of at least 10^{-8} M. Expressing their 5-HT_{1A} receptor antagonist activity (at both pre-synaptic and post-synaptic sites) as a function of dose, the compounds may have ID_{50} values of from 1 to 2000 μg/Kg, and preferably of from 1 to 200 μg/Kg.

As the compounds useful in the practice of the invention bind to 5-HT_{1A} receptors with an affinity at least 50 times greater, and preferably 100 times greater, than they bind to α_1-adrenergic receptors, they have α_1-adrenergic receptor binding constants in the micromolar range or weaker.

Medicaments prepared according to the invention are suitable for the treatment of neuromuscular disorders of the lower urinary tract, particularly those involving micturition, such as dysuria, incontinence, and enuresis. Without wishing to be bound by theory, it is believed that administration of 5-HT_{1A} receptor antagonists prevents unwanted activity of the sacral reflex arc and/or cortical mechanisms that control micturition. Thus it is contemplated that a wide range of lower urinary tract disorders can be treated using the compounds of the invention.

The compounds of the present invention may be formulated into liquid dosage forms with a physiologically acceptable carrier, such as phosphate buffered saline or deionized water. The pharmaceutical formulation may also contain excipients,

including preservatives and stabilizers, which are well-known in the art. The compounds can be formed into solid oral or non-oral dosage units such as tablets, capsules, powders, and suppositories, and may additionally include excipients, including without limitation lubricants, plasticizers, colorants, absorption enhancers, bactericides, and the like. Administration of medicaments prepared using true 5-HT_{1A} receptor antagonist compounds, their stereoisomers, pharmaceutically acceptable salts, hydrates or solvates, may be achieved by any effective route, including oral, enteral, intravenous, intramuscular, subcutaneous, transdermal, transmucosal (including rectal and buccal), and inhalation routes. Preferably, an oral or transdermal route is used (i.e., via solid or liquid oral formulations, or skin patches, respectively).

An effective amount of the medicament is an amount that results in measurable amelioration of at least one symptom of the disorder. This effective amount can be determined by experimentation known in the art, such as by establishing a matrix of dosages and frequencies and comparing a group of experimental units or subjects to each point in the matrix. Symptoms of urinary tract disorders include urgency, frequency, urine leakage, enuresis, dysuria, hesitancy, difficulty in emptying bladder. A measurable amelioration of any symptom or parameter is determined by a physician skilled in the art or reported by the patient to the physician.

For example, a single patient may suffer from several symptoms of dysuria simultaneously, such as, for example, urgency and frequency, either or both of which may be reduced using the methods of the present invention. In the case of incontinence, any reduction in the frequency or volume of unwanted passage of urine is considered a beneficial effect of the present methods of treatment.

The amount of the agent to be administered may range from about 0.01 to about 25 mg/kg/day, preferably from about 0.1 to 10 mg/kg/day and most preferably from about 0.2-5 mg/kg/day. It will be understood that the medicament formulations of the present invention need not in themselves contain the entire amount of the agent that is effective in treating the disorder, as such effective amounts can be reached by administration of a plurality of doses.

In a preferred embodiment of the invention, N-{2-[4-(2-methoxyphenyl)-1-piperazinyl]--ethyl}-N-(2-pyridyl)-cyclohexanecarboxamide (hereinafter Compound A) is formulated in capsules or tablets each containing from 50 to 200 mg of Compound A, and is administered to a patient at a daily dose of 200 mg for relief of urinary incontinence.

In another preferred embodiment of the invention, N-{2-[4-(4-indolyl)-1-piperazinyl]-ethyl}-N-(2-pyridyl)-cyclohexanecarboxamide (hereinafter Compound B) is formulated in capsules or tablets each containing from 20 to 60 mg of Compound B, and is administered to a patient at a daily dose of 60 mg for relief of urinary incontinence.

Compounds which possess the requisite pre-synaptic and post-synaptic 5-HT_{1A} antagonistic activity may be found amongst those having a protonatable nitrogen atom which is linked on one side to an aromatic or heteroaromatic ring and to a carbon chain on the other side. In addition, the protonatable nitrogen and the chain can form a ring. Compounds belonging to this general class may be tested according to the methods discussed below to determine whether they meet the other criteria for use in the invention.

Measurement of the specific binding activity of a compound towards different neuronal receptors (such as serotoninergic 5-HT_{1A} receptors, α_1- or α_2-adrenergic receptors, dopaminergic D_2 receptors, and serotoninergic 5-HT_2 receptors) may be achieved using any of a multiplicity of methods that are well known in the art, such as competitive binding to native or cloned receptors. Typically, a biological source of, for example, a 5-HT_{1A} receptor is used in which the receptor is present at a sufficiently high concentration so that labelled 5-HT or a labelled 5-HT_{1A} ligand is easily measurable. This source may comprise a mammalian tissue or fluid (either in situ or after removal) or a tissue culture cell. The target receptor may be expressed from either an endogenous gene or from a transfected receptor-encoding recombinant gene. For example, the rat hippocampus is a rich source of 5-HT_{1A} receptors. Alternatively, human 5-HT_{1A} receptor cDNA can be expressed in *E. coli* cells in culture as reported in Bertin B. et al., *J. Biol. Chem.*, 267, 8200 (1992). The ability of the test compound to compete with labelled 5-HT (or a labelled 5-HT_{1A} ligand) for receptor binding is then measured, and a binding constant is calculated using Scatchard analysis or equivalent computational methods well known in the art.

It will be understood that measurements of receptor binding affinity of a particular compound may vary depending upon the source of receptor, radiolabelled ligand, and other components, as well as specific assay conditions. Thus, Compound A is included in all assays as a standardization control. That is, the values of binding affinities obtained for Compound A are compared to values reported below in Example 2, i.e. $K_i = 3 \times 10^{-10}$ M for 5-HT_{1A} receptors and 3×10^{-7} M for α_1-adrenergic receptors, and the values obtained in the same assay for other test compounds are normalized proportionately.

Measurement of pre-synaptic and post-synaptic serotoninergic 5-HT_{1A} receptor antagonist activity may be achieved using neurophysiological assay methods. For example, Raphe cell firing measured electrophysiologically is used as an index of pre-synaptic 5-HT_{1A} receptor activity (VanderMaelen et al., *Brain Res.*, 289, 109, 1983). In this assay, a 5-HT_{1A} receptor agonist acting at pre-synaptic somatodendritic 5-HT_{1A} receptors inhibits Raphe neuronal firing, which is detected by measuring the electrical activity of 5-HT-containing neurons. Antagonists prevent the inhibitory action of the 5-HT_{1A} receptor agonist, resulting in the maintenance of high levels of serotonin in the synaptic cleft. An alternative system for measuring pre-synaptic activity is the inhibition of hypothermia induced in mice by 8-OH-DPAT (Moser, *Eur. J. Pharmacol.*, 193, 165, 1991).

Inhibition of adenylate cyclase activity in rat hippocampal slices is used as an indicator of post-synaptic 5-HT_{1A} receptor activity (Shenker et al.,*Eur.J.Pharmacol.*, 109, 427, 1985). In this assay, compounds exhibiting antagonistic activity at post-synaptic 5-HT_{1A} receptors antagonize the inhibitory effects of a 5-HT_{1A} agonist only on forskolin-stimulated adenylate cyclase activity and display no intrinsic effect on the basal activity of the enzyme. Alternative methods for measuring post-synaptic activity include inhibition of 8-OH-DPAT induced behavioural syndrome, in particular the forepaw treading symptom (Tricklebank et al., *Eur. J. Pharmacol.*, 117, 15, 1985). These and other methods are reviewed in Fletcher et al., *TiPS*, 14, 441, 1993.

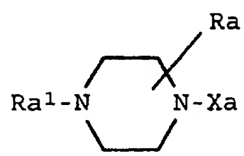
As discussed above, Compound A is included in all assays as a positive control; the values obtained for the pre-synaptic and post-synaptic antagonistic activity of Compound A are normalized proportionately with those disclosed below in Examples 6 and 7 (ID_{50} for pre-synaptic = 8.5 μg/Kg; post-synaptic = 14 μg/Kg), and the values obtained for other test compounds are compared.

Once a compound is identified as possessing 5-HT_{1A} receptor antagonist activity, its pharmacological activity is confirmed using one or more animal model systems for lower urinary tract disorders. Useful animal model systems include isovolumetric rhythmic bladder voiding contractions in anaesthetized rats and cystometry in conscious rats. In the first method, the urinary bladder is catheterized, ligated, and connected with a pressure recording device. The bladder is then filled until reflex voiding contractions occur, after which the frequency and amplitude of the voiding contractions are measured. In the second method, bladder volume capacity and micturition pressure are measured one day following bladder catheterization. In the first method, the test

compounds are administered intravenously prior to the measurements. Both oral and intravenous administration routes may be used in a second method. These methods are described in more detail in Examples 8 and 9 below, and were originally used to validate the predictive qualities of the true serotoninergic 5-HT_{1A} receptor antagonists for the foregoing urinary tract disorders.

5-HT_{1A} receptor antagonist compounds for use in the invention include compounds of the general formulae I to VII discussed below.

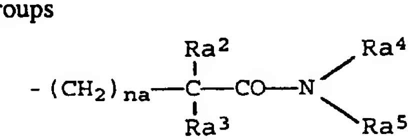
Piperazine derivatives of the general formula I



(I)

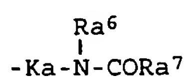
In these compounds:
Ra represents a hydrogen atom or a lower alkyl group;
Ra^1 represents an aryl, nitrogen-containing heteroaryl or nitrogen-containing bicyclic heteroaryl group; and
Xa represents one of the groups

(Aa)



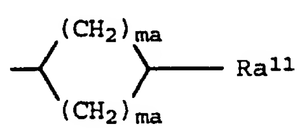
,

(Ba)



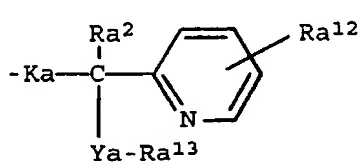
,

(Ca)



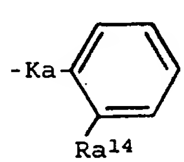
,

(Da)



and

(Ea)



;

na is 1 or 2;

ma is 1, 2 or 3;

Ra^2 represents a hydrogen atom or a lower alkyl group;

Ra^3 represents an aryl or aryl(lower)alkyl group;

Ra^4 represents a hydrogen atom or a C_1-C_3 alkyl group;

Ra^5 represents a hydrogen atom or a C_1-C_3 alkyl, C_3-C_{12} cycloalkyl or cycloalkyl(lower)alkyl group; or Ra^4 and Ra^5 together with the nitrogen atom to which they are attached represent a 1-azetidinyl, 1-pyrrolidinyl, piperidino, 1-perhydroazepinyl, morpholino, or 1-piperazinyl group, each optionally substituted by a lower alkyl, aryl or aryl(lower)alkyl group;

Ra^6 represents a monocyclic or bicyclic heteroaryl group;

Ra^7 represents a hydrogen atom, a lower alkyl, cycloalkyl, cycloalkenyl, cycloalkyl(lower)alkyl, aryl, aryl(lower)alkyl, heteroaryl or heteroaryl(lower)alkyl group, or a group $-NRa^8Ra^9$ or ORa^{10};

Ra^8 represents a hydrogen atom or a lower alkyl, aryl or aryl(lower)alkyl group;

Ra^9 represents a hydrogen atom or a lower alkyl, -CO-(lower)alkyl, aryl, -CO-aryl, aryl(lower)alkyl, cycloalkyl or cycloalkyl(lower)alkyl group; or Ra^8 and Ra^9 together with the nitrogen atom to which they are attached represent a saturated heterocyclic group which may contain an additional heteroatom;

Ra^{10} represents a lower alkyl, cycloalkyl, cycloalkyl -(lower)alkyl, aryl, aryl(lower)alkyl, heteroaryl or heteroaryl(lower)alkyl group;

Ra^{11} represents an aryl or nitrogen containing heteroaryl group;

Ra^{12} represents a hydrogen atom or a lower alkyl group;

Ra^{13} represents a hydrogen atom or a C_1-C_3 alkyl, C_3-C_{12} cycloalkyl or cycloalkyl(lower)alkyl group;

Ra^{14} represents an aryl group;

Ka represents a C_2-C_4 alkylene group optionally substituted by one or more lower alkyl groups; and

Ya represents a carbonyl, alkylene, hydroxyalkylene or hydroxycycloalkylene group or a group $-S(O)_{oa}$; where oa = 0 to 2.

When Ra^1 represents an aryl group, it preferably represents a phenyl group having a substituent in the ortho position or a 1-naphthyl group optionally substituted in the 2 or 7 positions. Examples of aryl groups Ra^1 are o-(lower)alkoxyphenyl, such as o-methoxyphenyl, or (lower)alkoxy substituted 1-naphthyl. When Ra^1 represents a bicyclic heteroaryl group, it preferably represents a 4-indolyl group.

When Ra^6 represents a bicyclic heteroaryl group, both rings can contain hetero ring atom(s), or only one ring can contain hetero atom(s). In the latter instance, the group Ra^6 is connected to the compound of formula (I) via the ring containing the hetero atom(s).

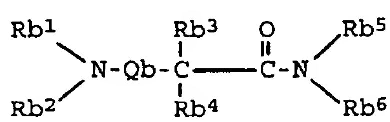
Examples of the heteroaryl group Ra^6 include monocyclic groups containing one hetero atom, such as pyridyl (particularly 2-pyridyl); monocyclic groups containing two hetero atoms, such as thiazolyl (particularly 2-thiazolyl); and bicyclic groups containing one or two hetero atoms, such as quinolinyl or isoquinolinyl and particularly 2-quinolinyl.

When Ra^{11} and Ra^{14} represent aryl groups, the preferred groups are phenyl. When Ra^{11} represents a heteroaryl group it is preferably a pyridyl group, optionally substituted by one or more alkyl groups.

Methods for the preparation of the piperazine derivatives I are disclosed in the following references: GB 2230780 (EP 395313), GB 2230781 (EP 395312), GB 2248836, (EP 481744), GB 2255337, GB 2262093, WO 94/15919, WO 94/15928, WO 94/21610, WO 95/33743 and GB 2277517.

Preferred piperazine derivatives I include:
1-{4-[4-(2-methoxyphenyl)-1-piperazinyl]-3-phenylbutanoyl}-perhydroazepine,
1-{4-[4-(2-methoxyphenyl)-1-piperazinyl]-2-phenylbutanoyl}-perhydroazepine,
1-[N-cyclohexylcarbonyl-N-(2-pyridyl)-2-aminoethyl)-4-(2-methoxyphenyl)-piperazine (Compound A),
1-[N-cyclohexylcarbonyl-N-(2-pyridyl)-2-aminoethyl)-4-(4-indolyl)-piperazine (Compound B),
1-[N-[2-(2-pyridylamino)-ethyl]-4-(2-methoxyphenyl)-piperazine (Compound C),
1-[N-[2-(2-pyridylamino)-ethyl]-4-(4-indolyl)-piperazine (Compound D), and
1-[2-(2-biphenyl)-ethyl]-4-(2-methoxyphenyl)-piperazine,
and their pharmaceutically acceptable acid addition salts.

<u>Compounds of the general formula II</u>



(II)

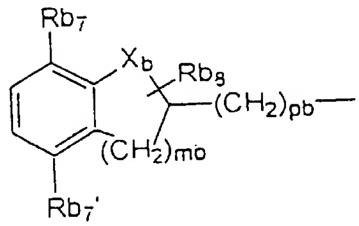
In these compounds:

Qb represents a C_1-C_3 alkylene group, optionally substituted by one or more lower alkyl groups;

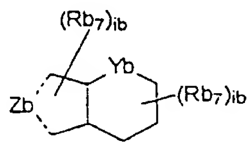
Rb^1 represents a hydrogen atom or a lower alkyl group;

Rb^2 represents one of the groups

(Ab)

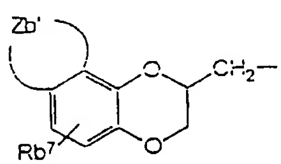


(Bb)



(Cb) Rb^9-CH_2CH_2- ,

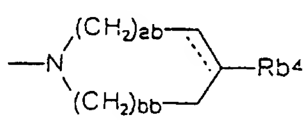
(Db)



(Eb) Rb^{10}-O-$CH_2CH(OH)CH_2$- and

(Fb) Rb^{10}-O-CH_2CH_2-;

or Rb^1 and Rb^2 together with the nitrogen atom to which they are attached represent a group of the formula



Rb^3 represents a hydrogen atom or a lower alkyl group;

Rb^4 represents an aryl, bicyclic aryl or heteroaryl group;

Rb^5 represents a hydrogen atom or a lower alkyl group;

Rb^6 represents a hydrogen atom or a C_1-C_{10} alkyl, C_3-C_{12} cycloalkyl, cycloalkyl(lower)alkyl, aryl or aryl(lower)alkyl group;

or Rb^5 and Rb^6 together with the nitrogen atom to which they are attached represent a saturated heterocyclic group, optionally containing an additional hetero atom and

optionally substituted by a halogen atom or a lower alkyl, aryl, aryl(lower)alkyl, lower alkoxy or halo(lower)alkyl group;

ab is 0 to 3 and bb is 0 to 3 but (ab + bb) is not more than 3;

---- represents an optional double bond which can be present provided that ab is at least 1;

Xb represents a group $-(CH_2)_{nb}-$, $-OCH_2-$ or $-SCH_2-$;

mb is 0 or 1, nb is 1 to 3, and pb is 0 or 1; provided that (mb + pb) is 1 and (mb + nb) is not more than 3;

Rb^7 represents a hydrogen or halogen atom, or a lower alkyl, (lower)alkylcarbonyl, lower alkoxy, (lower)alkoxycarbonyl, hydroxy, trifluoromethyl, carboxamido, nitro, cyano, amino, (lower)alkylamino or di(lower)alkylamino group;

$Rb^{7'}$ represents a hydrogen or halogen atom; with the proviso that when Xb represents a group $-OCH_2-$ or $-SCH_2-$ then $Rb^{7'}$ represents a hydrogen atom;

Rb^8 represents a hydrogen atom or a lower alkyl group;

ib = 0, 1 or 2; jb = 0, 1 or 2;

Yb represents an oxygen or sulphur atom or a methylene group;

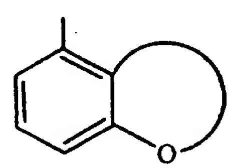
Zb represents the atoms necessary to form a heteroaromatic ring having from 5 to 7 carbon atoms fused to the non-aromatic ring containing the Yb group;

Rb^9 represents a monocyclic or bicyclic heteroaryl group;

Zb' represents either a pair of hydrogen atoms or the atoms necessary to form an aromatic or heteroaromatic ring fused to the benzodioxanyl group; and

Rb^{10} represents a monocyclic or bicyclic aryl or bicyclic heteroaryl group.

When Rb^4 represents a heteroaryl group, it is preferably a bicyclic oxygen-containing group of the formula:



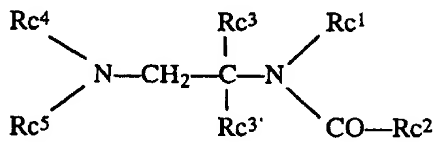
wherein the heterocyclic ring has from 5 to 7 ring atoms, is saturated or unsaturated, and optionally includes one or more hetero ring atoms or groups, such as -O-, -S-, $-SO_2-$ or NRb^3, in addition to the oxygen atom illustrated.

Examples of saturated heterocyclic groups which NRb^5Rb^6 may represent include 1-azetidinyl, 1-pyrrolidinyl, piperidino, 1-perhydroazepinyl, morpholino, 1-perhydroazocinyl and 1-piperazinyl groups.

The preparation of compounds II is disclosed in WO 94/03444 and WO 94/20481.

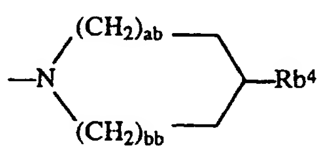
A preferred compound II is:
1-{4-[(1,4-benzodioxan-2-yl)-methylamino]-2-phenylbutanoyl}-perhydroazepine.

Compounds of the general formula III



(III)

In these compounds:
Rc^1 represents a heteroaryl or bicyclic heteroaryl group;
Rc^2 represents a cycloalkyl group;
Rc^3 represents a hydrogen atom or a lower alkyl group;
$Rc^{3'}$ represents a hydrogen atom or a lower alkyl group;
Rc^4 represents a hydrogen atom or a lower alkyl group; and
Rc^5 represents one of the groups (Ab), (Bb), (Cb), (Db), (Eb) and (Fb) as above defined;
or Rc^4 and Rc^5 together with the nitrogen atom to which they are attached represent a group of the formula

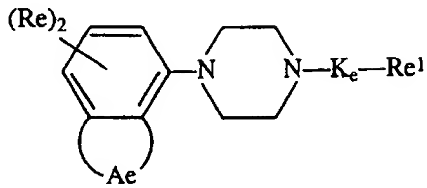


wherein ab, bb, Rb^4 and ---- are as above defined.

The compounds III and their methods of preparation are disclosed in WO 94/21611 and WO 95/02592.

Preferred compounds having formula III are:
N-(1,4-benzodioxan-2-ylmethyl)-N-methyl-N'-(2-pyridyl)-N'-cyclohexylcarbonyl--1,2-diaminoethane,
(R)-4-(2-methoxyphenyl)-1-[N-cyclohexanoyl-N-(2-pyridyl)-3-amino-2-propyl]--piperidine, and
(R)-4-(2-thienyl)-1-[N-cyclohexanoyl-N-(2-pyridyl)-3-amino-2-propyl]--1,2,3,6-tetrahydropyridine.

Compounds of the general formula IV



(IV)

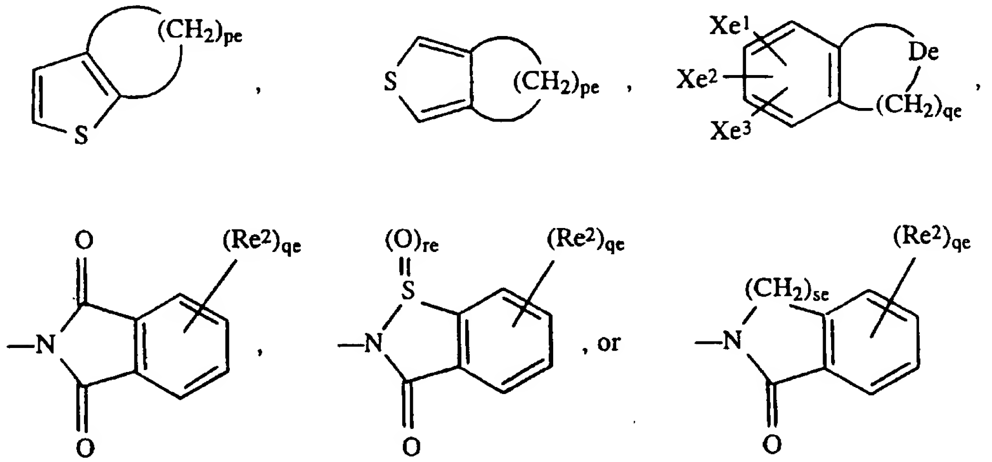
In these compounds:

Ae represents a group $-OCH=CH-$, $-OCH_2CH_2-$, $-OCH_2O-$, $-OCH_2CH_2O-$ or $-OCOCH=CH-$;

each Re independently represents a hydrogen or halogen atom or an alkyl, hydroxy, alkoxy, trifluoromethyl or cyano group;

Ke represents a C_1-C_8 linear or branched alkylene group, optionally substituted by an aryl or heteroaryl group;

Re^1 represents a phenyl, thienyl, naphthyl or benzothiophenyl group, or a group of the formula

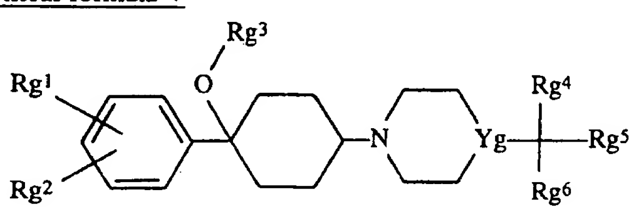


wherein pe is 3 or 4; qe is 0 to 3; re is 0 to 2; se is 1 or 2;
each Re^2 independently represents a halogen atom or an alkyl, hydroxy, alkoxy, trifluoromethyl or cyano group;
De represents a group -CH=CH- or $(CH_2)_{2-4}$; and
each of Xe^1, Xe^2 and Xe^3 independently represents a hydrogen atom or an alkyl, alkoxy, hydroxy, alkylthio, trifluoromethyl, nitro, amino or acetamido group, or two of Xe^1, Xe^2 and Xe^3 together represent a group $-OCH_2O-$ or $-OCH_2CH_2O-$.

The preparation of compounds having formula (IV) is disclosed in EP 490772, EP 574313 and EP 633260.

Preferred compounds having formula IV include:
1-[5-(1,4-benzodioxanyl)]-4-[3-(3-thienyl)-propyl]-piperazine,
1-[5-(1,4-benzodioxanyl)]-4-[2-(1-indanyl)-ethyl]-piperazine, and
1-[5-(1,4-benzodioxanyl)]-4-[3-(1-benzocyclobutyl)-propyl]-piperazine.

<u>Compounds of the general formula V</u>

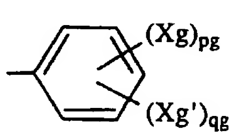


(V)

In these compounds:
each of Rg^1 and Rg^2 independently represents a hydrogen or halogen atom or a trifluoromethyl or C_1-C_4 alkoxy group;
or Rg^1 and Rg^2, being on adjacent carbon atoms, together represent a group of formula $-O(CH_2)_{ig}O-$ wherein ig is from 1 to 3;
each of Rg^3, Rg^4 and Rg^5 independently represents a hydrogen atom or a C_1-C_4 alkyl group or a phenyl group;
Yg represents a nitrogen atom or a group CH; and
Rg^6 represents a heteroaryl, phenyl or substituted phenyl group.

The preferred substituted phenyl groups which Rg^6 may represent have the formula Ag:

(Ag)



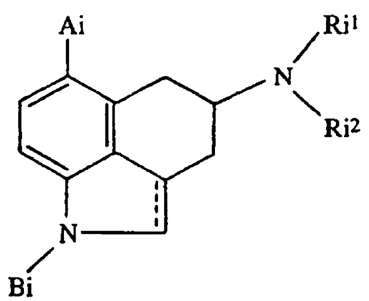
wherein pg is 0 to 5 and qg is 0 to 5 but (pg + qg) is not more than 5;
each of Xg and Xg' independently represents a halogen atom or a nitro, amino, carboxamido, C_1-C_4 alkyl, C_1-C_4 alkoxy, C_1-C_4 haloalkyl, C_1-C_4 alkylthio or the like; or Xg and Xg', being on adjacent carbon atoms, together represent a group $-O(CH_2)_{ng}O-$ wherein ng is 1 to 3.

The preferred heteroaryl groups which Rg^6 may represent include 3-pyridyl, 4-pyridyl, 2-thienyl, 2-furanyl and 1-methyl-2-pyrrolyl groups.

The preparation of compounds having formula V is disclosed in US 5387593 and EP 546583.

Preferred compounds having formula V include:
Z- and E-4-benzyl-1-[4-hydroxy-4-(1,4-benzodioxan-6-yl)-cyclohexyl]-piperazine, and
Z-4-(3-methoxybenzyl)-1-[4-methoxy-4-(1,3-benzodioxolan-5-yl)-cyclohexyl]-
-piperidine.

<u>Compounds of the general formula VI</u>



(VI)

In these compounds:
---- represents a single or a double bond;
Ri^1 represents a hydrogen atom, a C_1-C_4 alkyl, C_3-C_4 alkenyl, phenyl(C_1-C_4)alkyl or cyclopropylmethyl group, or a group $CORi^4$, $-(CH_2)_{ni}S(C_1\text{-}C_4)$alkyl or $-(CH_2)_{ni}C(O)NRi^9Ri^{10}$;
Ri^2 represents a hydrogen atom or a C_1-C_4 alkyl, C_3-C_4 alkenyl or cyclopropylmethyl group;
Ai represents a tetrazolyl or substituted tetrazolyl group, a heteroaryl group having 5 or 6 ring atoms of which from 1 to 3 may be oxygen, sulphur or nitrogen atoms, or a group $Qi{=}\underset{|}{C}{-}Xi$,

Bi represents a hydrogen atom, a C_1-C_4 alkyl group or an amino-blocking group;
Xi represents a hydrogen atom or a group -ORi^3, -SRi^3 or -NRi^{5}Ri^6;
Ri^3 represents a C_1-C_3 alkyl, substituted C_1-C_3 alkyl, aryl, substituted aryl, aryl(C_1-C_4)alkyl, substituted aryl(C_1-C_4)alkyl or C_3-C_7 cycloalkyl group;
Ri^4 represents a hydrogen atom or a C_1-C_4 alkyl, C_1-C_4 haloalkyl, C_1-C_4 alkoxy or phenyl group;
each of Ri^5 and Ri^6 independently represents a hydrogen atom or a C_1-C_4 alkyl, phenyl(C_1-C_4)alkyl or phenyl group;
or Ri^5 and Ri^6 together with the nitrogen atom to which they are attached represent a C_3-C_5 heterocyclic ring;
each of Ri^9 and Ri^{10} independently represents a hydrogen atom or a C_1-C_4 alkyl or C_5-C_8 cycloalkyl group;
ni is 1 to 4; and
Qi represents an oxygen or sulphur atom.

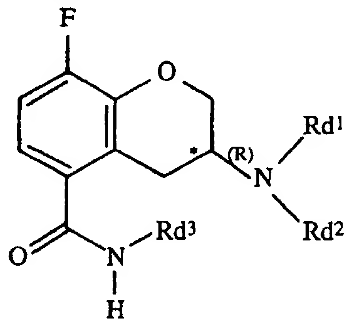
The term "amino-blocking group", as used herein, refers to a group which will prevent an amino group from participating in a reaction carried out on another functional group in the molecule, but which can be removed from the amine when desired. Such groups are described by T.W. Greene in chapter 7 of "*Protective Groups in Organic Synthesis*", John Wiley and Sons, New York, 1981. Groups which are useful for the compounds of the invention include benzyl and substituted benzyl groups such as 3,4-dimethoxybenzyl, o-nitrobenzyl and triphenylmethyl; groups having the formula COOR wherein R includes groups such as methyl, ethyl, propyl, isopropyl, 2,2,2-trichloroethyl, 1-methyl-1-phenylethyl, isobutyl, t-amyl, vinyl, allyl, phenyl, benzyl, p-nitrobenzyl, o-nitrobenzyl and 2,4-dichlorobenzyl; acyl and substituted acyl groups such as formyl, acetyl, chloroacetyl, dichloroacetyl, trichloroacetyl, trifluoroacetyl, benzoyl and p-methoxy-benzoyl; substituted sulphonyl groups such as methanesulphonyl, p-toluenesulphonyl, p-bromobenzene-sulphonyl, p-nitrophenylethyl and p-toluenesulphonyl-aminocarbonyl. Preferred amino-blocking groups are benzyl, acyl [C(O)R] or SiR_3 where R is C_1-C_4 alkyl, halomethyl or 2-halo-substituted-(C_2-C_4)alkoxy.

The preparation of compounds having formula VI is disclosed in EP 444854, EP 590971 and US 4576959.

Preferred compounds having formula VI include:
(2aS,4R)-4-(di-n-propylamino)-6-carbamoyl-1,2,2a,3,4,5-hexahydrobenz[c,d]indole,

(2aS,4R)-4-(di-n-propylamino)-6-dimethylcarbamoyl-
-1,2,2a,3,4,5-hexahydrobenz[c,d]indole,
(4R)-6-(5-isoxazolyl)-4-(di-n-propylamino)-1,3,4,5-tetrahydrobenz[c,d]indole,
(4R)-6-(2-oxazolyl)-4-(di-n-propylamino)-1,3,4,5-tetrahydrobenz[c,d]indole,
(4R)-6-(5-oxazolyl)-4-(di-n-propylamino)-1,3,4,5-tetrahydrobenz[c,d]indole, and
(4R)-6-[2-(1,3,4-oxadiazolyl)]-4-(di-n-propylamino)-1,3,4,5-tetrahydrobenz[c,d]indole.

Compounds of the general formula VII



(VII)

In these compounds:
Rd1 represents an n-propyl or cyclobutyl group;
Rd2 represents an isopropyl, t-butyl, cyclobutyl, cyclopentyl or cyclohexyl group; and
Rd3 represents a hydrogen atom or a methyl group.

Compounds of the general formula VII may be prepared according to the methods described in WO 95/11891.

Preferred compounds of the general formula VII include:
(R)-5-carbamoyl-3-(N,N-dicyclobutylamino)-8-fluoro-3,4-dihydro-2H-1-benzopyran,
(R)-3-[N-(t-butyl)-N-(n-propyl)-amino]-5-carbamoyl-8-fluoro-3,4-dihydro-
-2H-1-benzopyran, and
(R)-5-carbamoyl-3-(N-cyclobutyl-N-isopropylamino)-8-fluoro-3,4-dihydro-
-2H-1-benzopyran.

As used herein, lower alkyl indicates alkyl groups having from 1 to 6 carbon atoms and preferably from 1 to 4 carbon atoms. Lower alkenyl as used herein indicates alkenyl groups having from 2 to 6 carbon atoms and preferably from 2 to 4 carbon atoms. The cycloalkyl groups contain from 3 to 12 carbon atoms and preferably from 5 to about 7 ring atoms. Cycloalkyl groups also include bicyclic, tricyclic and tetracyclic groups, such as adamantyl.

As used herein, aryl refers to aromatic groups having from 6 to 12 carbon atoms, such as phenyl and naphthyl. These may be substituted by one or more substituents. Preferred substituents include halogen atoms and lower alkyl, lower alkoxy, halo(lower)alkyl, nitro, cyano, amido, (lower)alkoxycarbonyl; amino; (lower)alkylamino; and di(lower)alkylamino groups. Two adjacent substituents on the aromatic ring can together to form a further fused ring, e.g. benzodioxanyl. The term halogen refers to fluorine, chlorine, and bromine. The preferred halogens are chlorine and fluorine. Examples of the preferred aryl(lower)alkyl groups include benzyl and phenethyl, optionally substituted as described above.

As used herein, heteroaryl refers to an aromatic group containing one or more hetero atoms (e.g. oxygen, nitrogen, or sulphur) and which can be monocyclic or bicyclic. The monocyclic heteroaryl group refers to an aromatic ring containing one or more nitrogen or other heteroatoms, such as pyridyl, 2-thienyl, 2-furanyl, 1-methyl-2-pyrrolyl, pyrimidinyl, pyrazinyl, oxazolyl, thiazinyl and the like. Preferred heteroaryl groups include 2-pyridyl, 3-pyridyl and 4-pyridyl groups. The heteroaryl groups may be substituted by halogen atoms or lower alkyl, lower alkoxy, halo(lower)alkyl, nitro, amino, cyano, amido, (lower)alkoxycarbonyl, (lower)alkylamino, and di-(lower)alkylamino groups. Bicyclic heteroaryl refers to phenyl rings fused with a second ring containing one or more heteroatoms. A particularly preferred heteroatom is nitrogen. Examples of the bicyclic heteroaryl groups include indazolyl, quinolinyl, isoquinolinyl and indolyl. The bicyclic heteroaryl groups can be substituted by one or more substituents. A preferred bicyclic heteroaryl group is indolyl substituted with alkoxycarbonyl groups.

The following Examples illustrate the invention. Reference is made in the Examples to the accompanying drawings, of which:

Figure 1 is a graphic illustration of a typical recorder tracing showing the effect of Compound A on volume-induced contractions of anaesthetized rats, the arrow indicating the intravenous administration of 300 μg/kg of Compound A, and

Figure 2 is a graphic illustration of a typical recorder tracing showing the effect of Compound A on cystometrographic parameters in conscious rats, the arrow indicating oral treatment of the animal with 3 mg/kg of Compound A.

Example 1

1-[N-(2-pyridyl)-2-aminoethyl]-4-(2-methoxyphenyl)-piperazine (Compound C)

23.5 g of 1-(2-aminoethyl)-4-(2-methoxyphenyl)-piperazine [Hexachemie-Reuil Malmaison-France] and 4.85 ml of 2-chloropyridine were stirred at 160°C in a closed reaction vessel for 10.5 hours. The reaction mixture was cooled to room temperature, dissolved in 320 ml of chloroform and washed with 1N sodium hydroxide (3 x 320 ml), followed by water (2 x 400 ml). The organic layer was dried on sodium sulphate and then evaporated to dryness under reduced pressure. The crude product was purified by column flash chromatography eluting with an ethyl acetate : 3N NH_3 in methanol 100:2 mixture affording, after evaporation of the collected fractions, 5 g of the title compound as an oil. A sample was crystallized from ethyl acetate to give a solid melting at 89-94°C.

^{1}H-NMR ($CDCl_3$, δ)

8.08, ddd, 1H, CH at position 6 in the pyridine ring
7.40, ddd, 1H, CH at position 4 in the pyridine ring
6.80-7.05, m, 4H, 2-methoxyphenyl CHs
6.55, ddd, 1H, CH at position 5 in the pyridine ring
6.40, dd, 1H, CH at position 3 in the pyridine ring
5.10, bs, 1H, NH
3.85, s, 3H, CH_3O
3.38, dt, 2H, $C\underline{H_2}NH$
3.00-3.15, m, 4H, piperazine CHs
2.60-2.75, m, 6H, piperazine CHs and CH_2N
D_2O addition makes NH signal appear upfield as HDO.

Example 2

N-{2-[4-(2-methoxyphenyl)-1-piperazinyl]-ethyl}-N-(2-pyridyl)--cyclohexanecarboxamide . 2.66 HCl . 0.33 H_2O (Compound A)

To a solution of 4.26 g of the compound prepared in Example 1 in 42.5 ml of tetrahydrofuran, 6.52 ml of 2.5N n-butyllithium (hexane solution) was added dropwise at -22°C. After 40 minutes stirring at -20°C, 2.21 ml of cyclohexanecarbonyl chloride was added dropwise. The reaction mixture was stirred at -20°C for 20 minutes, then at room temperature for 3.5 hours. Water was cautiously added to quench the reaction, followed by 3N sodium hydroxide. Ethyl acetate extraction followed by washing the organic layer with water, drying on sodium sulphate and evaporating the solvent to dryness under reduced pressure gave an oily crude which was purified by column flash chromatography eluting with an ethyl acetate : 3N NH_3 in methanol 100:2 mixture. Evaporation of the collected fractions afforded 5 g of the title compound as a base, which was converted into the hydrochloride by dissolution in methanol and addition of excess 2.8N HCl in diethyl ether. Evaporation to dryness of the solvents and desiccation of the solid in vacuo yielded 5.30 g of the title compound. M.p. 161-164°C.

Elemental analysis for $C_{25}H_{34}N_4O_2$. 2.66 HCl . 0.33 H_2O:

calc. (%): C 57.14, H 7.16, N 10.66, Cl 17.95, H_2O 1.13

found (%): C 57.45, H 7.29, N 10.67, Cl 18.13, H_2O 1.20

^{1}H-NMR (D_6-DMSO, δ):

11.10-11.70, bs, 1H, NH+
8.58, dd, 1H, CH at position 6 in the pyridine ring
8.05, ddd, 1H, CH at position 4 in the pyridine ring
7.64, dd, 1H, CH at position 3 in the pyridine ring
7.45, dd, 1H, CH at position 5 in the pyridine ring
6.82-7.10, m, 4H, 2-methoxyphenyl CHs
5.20-5.80, bs, 2.4H, NH+ (remaining), H_2O
4.17, t, 2H, CH2NCO
3.79, s, 3H, CH_3O
3.00-3.75, m, 10H, CH_2N and piperazine CHs
2.15-2.35, m, 1H, cyclohexane CHCO
0.85-1.85, m, 10H, cyclohexane CHs
D_2O addition makes NH signals appear upfield as HDO.

Example 3
N-{2-[4-(4-Indolyl)-1-piperazinyl]-ethyl}-N-(2-pyridyl)-cyclohexanecarboxamide
(Compound B)

Step a:
N-(2,2-Dimethoxyethyl)-N-(2-pyridyl)-cyclohexanecarboxamide
13.2 ml of a 2.5M solution of butyl lithium in n-hexane was added to a solution of 6g of 2-(2-pyridylamino)-acetaldehyde dimethyl acetal (prepared as described in Beilstein E III/IV, *22*, 3871) in 40 ml of tetrahydrofuran stirred at 0°C and the resulting mixture was stirred at room temperature for 1 hour. 4.46 ml of cyclohexanecarbonyl chloride was then added dropwise over a period of 5 minutes. Stirring was continued for 5.5 hours and the reaction mixture was then evaporated to dryness *in vacuo*. The residue was purified by flash chromatography, eluting with chloroform:ethyl acetate 7:3, to afford 13.3 g of the title compound.

^{1}H-NMR ($CDCl_3$, δ):

8.48-8.54, m, 1H, CH at position 6 in the pyridine ring
7.75, ddd, 1H, CH at position 4 in the pyridine ring
7.18-7.44, m, 2H, CHs at positions 3 and 5 in the pyridine ring
4.65, t, 1H, $C\underline{H}CH_2$
3.90, d, 2H, CH_2
3.31, s, 6H, 2 x CH_3O
2.32, tt, 1H, $C\underline{H}(CH_2)_2$
0.80-1.85, m, 10H, cyclohexane CH_2s

Step b:
N-Formylmethyl-N-(2-pyridyl)-cyclohexanecarboxamide
A mixture of 1.46 g of N-(2,2-dimethoxyethyl)-N-(2-pyridyl)--cyclohexanecarboxamide, 0.05 g of 1,4-hydroquinone and 25ml of 2N HCl was stirred at 80°C for 20 minutes under a nitrogen stream. The mixture was then cooled to 0°C, diluted with 50ml of dichloromethane, and adjusted to pH 10 by addition of a 20% solution of sodium carbonate. The aqueous layer was extracted twice with dichloromethane and the combined organic layers were dried over anhydrous sodium sulphate. Evaporation to dryness *in vacuo* gave 0.94 g of the title compound, used without purification in the next reaction step.

^{1}H-NMR ($CDCl_3$, δ):

9.66, s, 1H, CHO
8.48-8.54, m, 1H, CH at position 6 in the pyridine ring
7.79, ddd, 1H, CH at position 4 in the pyridine ring
7.18-7.44, m, 2H, CHs at positions 3 and 5 in the pyridine ring
4.52, s, 2H, CH_2
2.48, tt, 1H, $C\underline{H}(CH_2)_2$
0.80-1.95, m, 10H, cyclohexane CH_2s

Step c:
N-{2-[4-(4-Indolyl)-1-piperazinyl]-ethyl}-N-(2-pyridyl)-cyclohexanecarboxamide HCl . $1.25H_2O$

A mixture of 0.94 g of N-formylmethyl-N-(2-pyridyl)-cyclohexanecarboxamide, 0.69 g of 1-(4-indolyl)-piperazine, 1.21 g of sodium triacetoxyborohydride, 0.44 ml of acetic acid and 30 ml of 1,2-dichloroethane was stirred at room temperature for 3 hours. The mixture was then diluted with 20 ml of water and adjusted to pH 10 by addition of a 20% solution of sodium carbonate. The aqueous layer was extracted twice with 1,2-dichloroethane and the combined organic layers were dried over anhydrous sodium sulphate. Evaporation to dryness *in vacuo* gave a crude product which was purified by flash chromatography, eluting with dichloromethane:methanol 98:2 to 95:5, to give 0.96 g of the base of the title compound. This was dissolved in 40 ml of dichloromethane and 3.8N hydrogen chloride in diethyl ether was added. The title compound precipitated and was filtered off. Yield 0.66 g. M.p. 181-187°C.

Example 4
1-(4-Indolyl)-4-[2-(2-pyridylamino)-ethyl]-piperazine . 3HCl . $2H_2O$ (Compound D)

Step a:
2-[4-(4-Indolyl)-1-piperazinyl]-N-(2-pyridyl)-acetamide

A mixture of 1.4 g of 1-(4-indolyl)-piperazine, 1.26 g of 2-chloro-N-(2-pyridyl)--acetamide (prepared as described in Beilstein E III/IV, *22*, 3881), 1.3 ml of diisopropylethylamine and 14 ml of dimethylformamide was stirred at 60°C under a nitrogen stream for 4 hours. The mixture was then diluted with 200 ml of water and extracted with ethyl acetate (4 x 50 ml). The combined organic layers were washed with water, dried on anhydrous sodium sulphate and evaporated to dryness *in vacuo* to give 2.37 g of the title compound as a crude base. Crystallization from methanol afforded 1.6 g melting at 198-201°C.

Step b:

1-(4-Indolyl)-4-[2-(2-pyridylamino)-ethyl]-piperazine . 3HCl . $2H_2O$

0.34 g of 95% lithium aluminium hydride was added to a solution of 2-[4-(4-indolyl)-1-piperazinyl]-N-(2-pyridyl)-acetamide in 30 ml of anhydrous tetrahydrofuran stirred at room temperature. The resulting mixture was stirred under reflux for 10 hours, and subsequently cooled. It was then diluted with 7ml of 2N sodium hydroxide and 50ml of water and dried on anhydrous sodium sulphate. The solvents were evaporated off *in vacuo*, giving 0.94 g of an oily residue. Purification by flash chromatography, eluting with ethyl acetate:methanol 96:4 to 70:30, gave 0.76 g of the base of the title compound. This was dissolved in 20 ml of dichloromethane and an excess of 3.8N hydrogen chloride in diethyl ether was added. The title compound precipitated. It was filtered off and dried at 60°C (0.5 mm Hg). M.p. (127) 144-152°C.

Example 5

Measurement of Binding of Test Compounds to 5-HT_{1A} and α_1-Adrenergic Receptors

[^{3}H] prazosin binding (α_1-receptors):

Rat cerebral cortices were homogenized in 50 volumes of ice-cold 50 mM Tris-HCl pH 7.4. The homogenates were centrifuged at 48,000 x g for 10 minutes, and the pellets were resuspended in the same volume of ice-cold buffer, centrifuged, and resuspended two more times. The final pellets were resuspended in 100 volumes of 50 mM Tris-HCl, pH 7.4, containing 0.1% ascorbic acid and 10 μM pargyline. 1-ml samples were incubated for 30 min at 25°C with 0.35 nM [^{3}H]prazosin, in the absence or presence of different concentrations (10^{-5} to 10^{-10} M) of the test compound. Non-specific binding was determined in the presence of 10 μM phentolamine. The incubations were terminated by rapid filtration through Whatman GF/B filters using a Brandel cell harvester, after which the filters were washed with 3x3 ml of ice-cold buffer. The radioactivity retained on the filters was determined by liquid scintillation counting. The results are shown in Table 1 below.

[^{3}H]8-OH-DPAT binding ($5HT_{1A}$ receptors):

Rat hippocampi were homogenized in 50 volumes of ice-cold 50 mM Tris-HCl pH 7.4. The homogenates were centrifuged at 48,000 x g for 10 minutes, and the pellets were resuspended in the same volume of ice-cold buffer, incubated for 10 minutes at 37°C, centrifuged and resuspended two more times. The final pellets obtained were resuspended in 100 volumes of 50 mM Tris-HCl, pH 7.4, containing 0.1% ascorbic

acid and 10 μM pargyline. 1 ml samples were incubated for 30 min at 25°C with 1 nM [^{3}H]8-OH-DPAT, in absence or presence of different concentrations (10^{-5} to 10^{-10} M) of the test compound. Non-specific binding was determined in the presence of 10 μM 5-HT. The incubations were terminated by rapid filtration through Whatman GF/B filters using a Brandel cell harvester, after which the filters were washed with 3 x 3 ml of ice-cold buffer. The radioactivity retained on the filters was determined by liquid scintillation counting. The results are shown in Table 1 below.

TABLE 1

Binding affinity for the 5-HT_{1A} receptor and α_1-adrenergic receptor.

Data are expressed as Ki (nM).

Compound	5-HT_{1A} receptor	α_1-adrenergic receptor
Compound A	0.3	295.5
Compound B	0.13	458.3
Compound C	20.2	214.7
Compound D	16.3	89.2
NAN-190	1.9	4.8

These results indicate that Compound A and Compound B bind tightly and selectively to the 5-HT_{1A} receptor. By contrast, NAN-190 exhibits nearly equivalent binding to both receptors.

Example 6

Measurement of Pre-Synaptic 5-HT_{1A} Receptor Antagonist Activity

Antagonism of hypothermia induced by 8-OH-DPAT in mice:

The antagonistic effect of 5-HT_{1A} receptor antagonists on hypothermia induced by 8-OH-DPAT was evaluated by the method of Moser (Moser, *Eur. J. Pharmacol.*, 193, 165, 1991) with minor modifications.

Male CD-1 mice (28-38 g) obtained from Charles River (Italy) were housed in a climate-controlled room (temperature 22 ± 2°C; humidity 55 ± 15%) and maintained on a 12 h light/dark cycle with free access to food and water. On the day of experiment, mice were placed singly in clear plastic boxes under the same ambient

conditions. Body temperature was measured by the insertion of a temperature probe (Termist TM-S, LSI) into the rectum to a depth of 2 cm. Rectal temperature was measured immediately prior to subcutaneous injection of the test compound and 30 min later. All animals then received 8-OH-DPAT (0.5 mg/kg s.c.) and their temperature was measured 30 min later. For each animal, temperature changes were calculated with respect to pretreatment values and the mean values were calculated for each treatment group.

A linear regression equation was used in order to evaluate ID_{50} values, defined as the dose of antagonist needed to block 50% of the hypothermic effect induced by 0.5 mg/kg 8-OH-DPAT administered subcutaneously.

The results are shown in Table 2 below.

TABLE 2

Antagonistic activity for the pre-synaptic 5-HT_{1A} receptor.

COMPOUND	ID_{50} (95%) C.L. in μg/kg s.c.
Compound A	8.5 (5.8-12.5)
Compound B	1.9 (1.0-3.7)
NAN-190	not active

These results demonstrate that Compound A and Compound B have significant pre-synaptic 5-HT_{1A} receptor antagonist activity.

Example 7

Measurement of Post-Synaptic 5-HT_{1A} Receptor Antagonist Activity

Inhibition of forepaw treading induced by 8-OH-DPAT in rats:

The inhibitory effect of 5-HT_{1A} receptor antagonists on the forepaw treading induced in rats by subcutaneous injection of 8-OH-DPAT was evaluated by the method of Tricklebank (Tricklebank et al., *Eur.J. Pharmacol.,* 117: 15, 1985) with minor modifications.

Male Sprague-Dawley rats (150-175 g) obtained from Charles River (Italy), were housed in a climate-controlled room and maintained on a 12 h light/dark cycle with free access to food and water. On the day of experiment, rats were placed singly in clear plastic boxes. Reserpinised rats were treated with reserpine, 1 mg/kg s.c., 18-24

h before the test. For evaluation of antagonistic activity, compounds were i.p. or s.c. administered 16 min before 8-OH-DPAT (1 mg/kg s.c.). Observation sessions of 30 s duration began 3 min after treatment with the agonist and were repeated every 3 min over a period of 15 min.

The appearance of the forepaw treading symptom induced by post-synaptic stimulation of the $5HT_{1A}$ receptors was noted, and its intensity was scored using a ranked intensity scale in which: 0 = absent, 1 = equivocal, 2 = present and 3 = intense. Behavioural scores for each treated rat were accumulated over the time course (5 observation periods) and expressed as mean values of 8-10 rats.

A linear regression equation was used in order to evaluate ID_{50} values, defined as the dose of antagonist needed to block 50% of the forepaw treading intensity induced by 1 mg/kg 8-OH-DPAT administered subcutaneously.

The results are shown in Table 3 below.

TABLE 3

Compound	NORMAL RATS ID_{50} $\mu g/Kg$	RESERPINIZED RATS ID_{50} $\mu g/Kg$
Compound A (s.c.)	14 (12-16)	8.5 (5.8-12.5)
NAN-190 (i.p.)	700 (500-1000)	2000 (1600-2400)

These results demonstrate that Compound A exhibits significant post-synaptic $5\text{-}HT_{1A}$ receptor antagonist activity. NAN-190, by contrast, is much less active. Taken together, the bioassays for pre-synaptic and post-synaptic antagonist activity are effective for identifying compounds that exhibit both activities at levels that render them useful in treating urinary tract disorders.

Example 8

Effect of $5\text{-}HT_{1A}$ Receptor Antagonists on Volume-Induced Rhythmic Bladder Voiding Contractions in Anaesthetized Rats

Female Sprague Dawley rats weighing 225-275 g (Crl: CD° BR, Charles River Italia) were used. The animals were housed with free access to food and water and were maintained on a forced 12 h alternating light-dark cycle at 22-24°C for at least one week, except during the experiment. The activity on the rhythmic bladder voiding

contractions was evaluated according to the method of Dray (*J. Pharmcol. Methods*, 13:157, 1985), with some modifications as in Guarneri (*Pharmacol. Res.*, 27:173, 1993). Briefly, rats were anaesthetized by subcutaneous injection of 1.25 g/kg (5 ml/kg) urethane, after which the urinary bladder was cathetized via the urethra using PE 50 polyethylene tubing filled with physiological saline. The catheter was tied in place with a ligature around the external urethral orifice and was connected with conventional pressure transducers (Statham P23 ID/P23 XL). The intravesical pressure was displayed continuously on a chart recorder (Battaglia Rangoni KV 135 with DC1/TI amplifier). The bladder was then filled via the recording catheter by incremental volumes of warm (37°C) saline until reflex bladder voiding contractions occurred (usually 0.8-1.5 ml). Two parameters were recorded from the cystometrogram: the frequency of voiding contractions, calculated by counting the number of peaks/15 min of observation, and the mean amplitude of these contractions (mean height of the peaks in mmHg) in the same time period. For intravenous (i.v.) injection of bioactive compounds, a PE 50 polyethylene tubing filled with physiological saline was inserted into the jugular vein.

Bioactivity was assessed in individual animals (using 6-10 rats per dose), by recording the number and height of the peaks for 15 min after drug injection and comparing them with those previously recorded for 15 min after the intravenous administration of vehicle alone. In the evaluation of the mean amplitude of peaks after treatment, only the results from the cystometrograms showing a reduction in the frequency of contractions of $\leq 50\%$ were utilized. The statistical significance of changes in frequency and amplitude before and after treatment was assessed by Student's *t* test for paired data. Changes showing a probability $P<0.01$ were considered significant.

An all-or-none criterion was also used to compare bioactivity in terms of ED_{50} values. Rats showing a treatment-related change of $\geq 30\%$ relative to the basal value were considered to be positive. Quantal dose-response curves and ED_{50} values were evaluated by the method of Bliss (*J. Pharm. Pharmacol.*, 11:192, 1938). In addition, since most compounds produced an effect that was relatively rapid in onset and led to a complete cessation of bladder contractions (as shown in Figure 1), bioactivity was conveniently estimated by measuring the duration of bladder quiescence (i.e., the duration of time during which no contractions occurred). To compare the potency of the tested compounds in inhibiting the frequency of the bladder voiding contractions, equieffective doses producing 10 minutes of disappearance time (ED_{10min}) were computed by means of least square linear regression analysis.

The rapid distension of the urinary bladder in urethane-anaesthetized rats produced a series of rhythmic bladder voiding contractions whose characteristics have been described (Maggie et al., *Brain Res.*, 380, 83, 1986; Maggi, et al., *J. Pharmacol. Exp. Ther.*, 230, 500, 1984). The frequency of these contractions is related to the sensory afferent arm of reflex micturition and to the integrity of the micturition centre, while their amplitude is a property of the efferent arm of the reflex (Maggi et al., *J. Pharmacol. Meth.*, 15, 157, 1986; Maggi et al., *Brain Res.*, 415, 1, 1987; Maggi et al., *Naun. Schmied. Arch. Pharmacol.*, 332, 276, 1986; Maggi et al., *J. Urol.*, 136, 696, 1986). In this model system, compounds that act mainly on the CNS (such as morphine) cause a reduction in the voiding frequency, whereas drugs that act at the level of the detrusor muscle lower the amplitude of the bladder contractions.

The results are tabulated in Tables 4 and 5 below.

TABLE 4

Effects on rhythmic bladder voiding contractions after intravenous administration.

Data represent the mean values ± S.E. of the number of contractions observed before and after the i.v. administration of the tested compounds, as well as the amplitude of the peaks recorded in animals showing a reduction of the frequency <50%. The ED50 (and 95% confidence limits) values were evaluated on the basis of a quantal criterion as described in the Methods.

COMPOUND Dose μg/kg i.v.	No. of rats	FREQUENCY No. contr./15 min before treatment	FREQUENCY No. contr./15 min after treatment	AMPLITUDE mm Hg before treatment	AMPLITUDE mm Hg after treatment
Compound A					
1	10	11.7±1.0	11.6±1.3	25.4±2.0	23.1±1.9*
3	10	11.5±0.7	9.1±1.0*	25.1±2.2	21.7±2.0*
10	10	11.5±1.5	5.9±1.6*	26.0±3.8	22.3±3.8
30	10	11.8±0.7	3.8±0.7*	28.5±2.5	25.0±4.0
100	10	12.0±0.9	4.1±1.1*	28.0±4.6	25.7±2.9
300	10	9.5±0.6	2.2±0.5*	n.c.	n.c.
ED_{50}(μg/kg)		5 (3 ÷ 8)		n.a.	
Compound B					
0.3	6	10.7±1.4	11.3±1.7	31.0±3.7	27.7±4.1
1	6	11.7±1.5	8.7±1.3	26.5±4.3	22.7±5.1*
3	6	9.7±0.8	4.7±1.1*	33.7±2.4	25.3±1.2
10	6	11.7±1.7	4.2±0.9*	19.0	17.0
30	6	12.0±1.1	4.5±1.4	n.c.	n.c.
ED_{50}(μg/kg)		1 (0.6 ÷ 2)		n.a.	

TABLE 4 continued

COMPOUND Dose µg/kg i.v.	No. of rats	FREQUENCY No. contr./15 min before treatment	after treatment	AMPLITUDE mm Hg before treatment	after treatment
Compound C					
30	6	9.7±1.0	7.2±1.3	29.6±7.4	24.0±6.2
100	6	9.7±1.0	4.5±1.6*	19.0±0.0	15.5±0.5
300	6	12.7±1.3	2.2±0.9*	n.c.	n.c.
1000	6	11.2±1.0	4.0±1.4*	40.0±0.0	25.5±0.5
ED_{50}(µg/kg)		54 (33 ÷ 91)		n.a.	
Compound D					
30	6	9.3±0.8	9.5±1.3	27.0±2.4	23.8±2.6*
100	6	15.3±1.0	9.3±2.8	27.0±1.5	23.7±1.5*
300	6	13.2±2.7	4.2±2.2*	26.0	20.0
1000	6	10.2±0.6	4.2±1.5*	32.0±5.0	23.5±5.5
ED_{50}(µg/kg)		77 (49 ÷ 122)		n.a.	
Flavoxate					
300	5	9.2±1.2	8.8±1.7	25.9±1.3	24.1±1.8
1000	17	10.1±0.7	8.6±0.9	26.1±2.3	25.0±2.2
3000	21	10.7±0.7	6.5±0.7*	18.9±0.9	16.6±1.2
10000	20	11.3±0.8	5.5±0.6*	19.9±1.6	19.2±1.5
ED_{50}(µg/kg)		2650 (1430 ÷ 4910)		n.a.	
Oxybutynin					
30	6	14.8±1.9	15.5±2.3	26.7±2.7	23.3±2.5
100	6	13.3±1.1	14.7±1.0	25.0±3.7	18.7±2.7*
300	12	10.1±0.7	8.8±0.8	20.9±1.7	13.6±0.8*
1000	13	9.7±0.8	9.5±0.8	20.3±2.4	11.8±1.2*
3000	13	10.0±0.7	9.9±1.5	18.0±1.4	10.8±1.0
ED_{50}(µg/kg)		n.a.		240 (140 ÷ 400)	
NAN-190(A)					
30	10	13.5±1.2	11.8±1.5	30.2±3.7	26.1±3.1*
100	10	13.6±1.0	6.4±1.1*	22.5±1.7	17.3±2.9
300	11	11.8±1.1	6.6±1.1*	24.1±1.8	17.6±1.7*
ED_{50}(µg/kg)		46 (23 ÷ 92)		n.a.	

* = $p \leq 0.01$ (Student's *t* test for paired data)
n.c. = not calculated
n.a. = not active on the parameter
A) = higher doses were not tested because of the high toxicity and low solubility of this compound.

TABLE 5

Effects on rhythmic bladder voiding contractions after intravenous administration.

Data represent the mean values ± S.E. of the duration of bladder quiescence (disappearance time of contractions in min). The ED_{10min} values represent the extrapolated dose inducing 10 min of disappearance of the contractions.

COMPOUND Dose μg/kg i.v.	No. of rats	BLADDER CONTRACTIONS Disappearance time (min)
Compound A		
1	10	1.34 ± 0.23
3	10	2.15 ± 0.42
10	10	8.13 ± 1.90
30	10	8.87 ± 1.08
100	10	12.56 ± 2.07
300	10	13.37 ± 1.83
ED_{10min}(μg/kg)		37 (18 ÷ 77)
Compound B		
0.3	6	1.10 ± 0.16
1	6	4.33 ± 1.30
3	6	7.58 ± 2.15
10	6	10.00 ± 0.92
30	6	8.85 ± 1.53
ED_{10min}(μg/kg)		9 (3 ÷ 24)
Compound C		
30	6	4.00 ± 1.87
100	6	9.60 ± 2.37
300	6	12.37 ± 2.63
1000	6	14.00 ± 4.45
ED_{10min}(μg/kg)		173 (28 ÷ 1087)
Compound D		
30	6	1.63 ± 0.50
100	6	6.55 ± 2.24
300	6	12.75 ± 2.45
1000	6	9.37 ± 2.44
ED_{10min}(μg/kg)		181 (89 ÷ 366)

TABLE 5 continued

COMPOUND Dose µg/kg i.v.	No. of rats	BLADDER CONTRACTIONS Disappearance time (min)
Flavoxate		
300	5	1.70 ± 0.60
1000	17	3.04 ± 0.96
3000	21	5.30 ± 1.00
10000	20	8.25 ± 1.90
ED_{10min}(µg/kg)		> 10000
NAN-190(A)		
30	10	1.80 ± 0.52
100	10	6.34 ± 1.18
300	11	5.47 ± 1.93
ED_{10min}(µg/kg)		> > 300

A) = higher doses were not tested because of the high toxicity and low solubility of this compound.

Compound B, after intravenous administration, dose dependently inhibited the frequency of the rhythmic bladder voidings and also reduced amplitude to some extent. Compound A, after intravenous administration, dose dependently inhibited the frequency of the rhythmic bladder voidings with no effect on their amplitude. The maximal change of this parameter, in fact, was about 13% and no dose-dependence was observed. Of the test compounds shown, Compound B was the most potent, being 46- and 2650-fold more active than NAN-190 and flavoxate, respectively. Compound A was 9- and 530-fold more active than NAN-190 and flavoxate, respectively.

By contrast, oxybutynin was only effective at reducing the amplitude of the contractions, confirming that its effects are due to a complete inhibition of the muscarinic receptors in the bladder.

The compounds that reduced the contraction frequency induced a complete and transient disappearance of contractions for a time period that was directly proportional to the administered dose (Table 5).

Tables 4 and 5 also illustrate the effects on volume-induced bladder contractions of flavoxate, a drug widely utilized in clinical therapy for bladder dysfunctions. Administration of this drug resulted in suppression of bladder contractions. The mean disappearance time observed after administration of the highest tested dose (10,000 μg/kg i.v.) was 8.25 $\pm$ 1.90 min. NAN-190 at the highest tested doses of 100-300 μg/kg gave a maximum disappearance time ranging from 5.5 to 6.3 min. (higher doses were not tested because of the high toxicity and low solubility of this compound).

These results indicate that Compound B and Compound A are potent compounds in reducing the frequency of the voiding contractions when compared to flavoxate and NAN-190 in terms of both absolute potency (ED_{50}) and disappearance time (ED_{10min}). Their mechanism of action appears to be different from that of oxybutynin, a peripheral antimuscarinic. Furthermore, their effects appeared at very low doses.

Example 9

Effect of 5-HT_{1A} Receptor Antagonists on Cystometric Parameters in the Conscious Rat

A. Methods:

Male Sprague Dawley rats (Crl: CD° BR) weighing 250-350 g were used. The animals were housed with free access to food and water and maintained on a forced 12 h alternating light-dark cycle at 22-24°C for at least one week, except during performance of the experiment.

To quantify urodynamic parameters in conscious rats, cystometrographic studies were performed using procedures described in Pietra et al., *IRCS Med. Sci.*, 14, 992, 1986; and Guarneri et al., *Pharmacol. Res.*, 24, 175, 1991.

Male rats were anaesthetized with nembutal (30 mg/kg) and chloral hydrate (125 mg/kg) i.p. and were placed in a supine position. An approximately 10 mm long midline incision was made in the shaved and cleaned abdominal wall. The urinary bladder was gently freed from adhering tissues, emptied, and then cannulated, via an incision at the dome, with a polyethylene cannula (Portex PP30), which was permanently sutured with silk thread. The cannula was exteriorized through a subcutaneous tunnel in the retroscapular area, where it was connected with a plastic adapter to avoid the risk of removal by the animal. For intravenous (i.v.) injection of test compounds, a PE 50 polyethylene tubing filled with physiological saline was inserted into the jugular vein and exteriorized in the retroscapular area.

Since cystometrographic parameters have been reported to be influenced by the time elapsed after catheter implantation Yaksh et al. *Amer. J. Physiol.*, 251, R1177, 1986 the rats were utilized exclusively one day after implantation.

On the day of the experiment, the rats were placed in Bollman's cages; after a stabilization period of 20 min, the free tip of the bladder catheter was connected through a T-shaped tube to a pressure transducer (Bentley T 800/Marb P 82) and to a peristaltic pump (Gilson minipuls 2) for a continuous infusion, at the constant rate of 0.1 ml/min, of saline solution into the urinary bladder. The intraluminal pressure signal during infusion was continuously recorded on a polygraph (Battaglia Rangoni KO 380 with ADC1/T amplifier). Two urodynamic parameters were evaluated: bladder volume capacity (BVC) and micturition pressure (MP). BVC (in ml) is defined as the minimum volume infused after which detrusor contraction (followed by micturition) occurs. MP (in mmHg) is defined as the maximal intravesical pressure induced by the contraction of detrusor during micturition. Basal BVC and MP values were calculated as the means of the first two recorded cystometrograms. At this point, the infusion was interrupted and the test compounds were administered. Fifteen minutes after intravenous administration, or one hour after oral drug administration, two additional cystometrograms were recorded in each animal and the mean values of the two cystometrographic parameters were calculated. A typical tracing is shown in Figure 2, where the effects of 3 mg/kg p.o. of Compound A are shown.

The statistical significance of the differences in urodynamic parameter values was evaluated by Student's *t* test for paired data. Only changes showing a probability $P<0.01$ were considered to be significant.

B. Results:

The effects on urodynamic parameters in conscious rats after i.v. administration of different doses of Compound A and the reference compounds are summarized in Tables 6 and 7.

TABLE 6

Effects on cystometrogram in conscious rats.

Data represent the mean ± S.E. values (ml) of bladder volume capacity (BVC), before and 15 min after i.v. injection of the compounds.

COMPOUND Dose μg/kg i.v.	No. of rats	BVC before treatment	 after treatment	 % change
CONTROL vehicle	12	0.50±0.09	0.43±0.06	-17
Compound A				
100	8	0.65±0.06	0.66±0.08	+4
300	9	0.47±0.05	0.63±0.06*	+32
1000	20	0.64±0.05	0.83±0.07*	+29
2000	10	0.48±0.04	0.63±0.05*	+32
Flavoxate				
300	17	0.76±0.11	0.87±0.11	+14
1000	14	0.88±0.15	1.11±0.16*	+26
3000	18	0.77±0.08	1.07±0.12*	+39
Oxybutynin				
100	13	0.82±0.15	0.89±0.18	+9
300	12	0.83±0.13	0.83±0.12	0
1000	12	0.94±0.19	1.00±0.18	+7
NAN-190(A)				
30	8	0.74±0.09	0.78±0.10	+6
100	8	0.68±0.10	0.76±0.10	+12
300	8	0.62±0.06	0.61±0.06	-1

* = $p \leq 0.01$ (Student's *t* test for paired data)

A) = higher doses were not tested because of the high toxicity and low solubility of this compound.

TABLE 7

Effects on cystometrogram in conscious rats.

Data represent the mean values ± S.E. (mmHg) of micturition pressure (MP), before and 15 min after i.v. injection of the compounds.

COMPOUND Dose μg/kg i.v.	No. of rats	MP before treatment	MP after treatment	% change
CONTROL vehicle	12	91.3± 9.2	87.9± 9.9	-4
Compound A				
100	8	93.0± 8.3	83.8± 8.7	-10
300	9	78.7± 5.8	70.0± 4.1	-11
1000	20	104.6± 6.4	91.0± 6.3*	-13
2000	10	101.8±10.9	81.5±14.1	-20
Flavoxate				
300	17	89.2±10.7	95.0±10.9	+7
1000	14	90.4±10.7	80.1±11.1	-12
3000	18	72.6± 9.3	75.2±9.5	+4
Oxybutynin				
100	13	95.2± 9.2	77.4±10.3*	-19
300	12	82.3± 8.7	50.5± 6.3*	-39
1000	12	110.9±10.2	29.6± 5.6*	-73
NAN-190(A)				
30	8	99.4±10.1	104.6± 9.7	+5
100	8	93.8±11.5	82.5± 9.2	-12
300	8	86.6±10.3	88.4±11.8	+2

* = $p \leq 0.01$ (Student's *t* test for paired data)

A) = higher doses were not tested because of the high toxicity and low solubility of this compound.

The administration of Compound A induced constant and significant increases of the BVC. Flavoxate (1000-3000 μg/kg) also induced increases in BVC, and the differences between basal and after treatment values were statistically significant (Table 6).

Oxybutynin was inactive on BVC (Table 6), but induced very consistent, significant and dose-related reductions of MP (the approximate ED_{50} value was 400 μg/kg), in contrast to Compound A and flavoxate which were inactive on this parameter (Table 7). NAN-190 was devoid of significant effects on both parameters up to the highest administrable dose of 300 μg/kg.

The effects of these compounds after oral administration were also tested. The results are shown in Tables 8 and 9 below.

TABLE 8

Effects on cystometrogram in conscious rats.

Data represent the mean values ± S.E. (ml) of bladder volume capacity (BVC), before and 1 hour after oral administration of the compounds.

COMPOUND Dose mg/kg p.o.	No. of rats	BVC before treatment	BVC after treatment	% change
CONTROL vehicle	11	0.64±0.10	0.73±0.13	+14
Compound A				
1	10	0.52±0.07	0.60±0.08	+15
3	10	0.67±0.07	0.91±0.10*	+35
10	10	0.54±0.06	0.73±0.10*	+37
Oxybutynin				
1	8	0.56±0.11	0.74±0.11*	+31
3	8	0.54±0.07	0.63±0.13	+18
10	8	0.55±0.08	0.70±0.11	+27
NAN-190				
10	10	0.54±0.08	0.46±0.07	-14
30	10	0.71±0.09	0.60±0.09	-15

* = p ≤0.01 (Student's *t* test for paired data)

TABLE 9

Effects on cystometrogram in conscious rats.

Data represent the mean values ± S.E. (mmHg) of micturition pressure (MP), before and 1 hour after oral administration of the compounds.

Compound Dose mg/kg p.o.	No. of rats	MP before treatment	MP after treatment	% change
CONTROL vehicle	11	84.1±10.1	73.3±11.0	-13
Compound A				
1	10	96.0±8.4	93.7±7.2	-2
3	10	112.5±6.5	107.6±9.2	-4
10	10	90.2±7.1	86.6±7.6	-4
Oxybutynin				
1	8	92.1±13.3	77.3±9.8	-16
3	8	82.1±5.1	42.1±5.1*	-49
10	8	98.3±9.0	31.8±3.9*	-68
NAN-190				
10	10	106.1±10.4	90.8±12.5	-14
30	10	105.1±10.5	95.8±15.3	-9

* = p ±0.01 (Student's *t* test for paired data)

Compound A produced a significant increase of BVC after oral administration of 3 mg/kg, and no changes in MP values were detected. Oxybutynin caused a significant increase of the BVC after oral administration at the lowest utilized dose (1 mg/kg), and produced a dose-related reduction of the MP values that was consistent and significant with 3 and 10 mg/kg dose-levels (approximate ED_{50} value was 4 mg/kg). NAN-190 was inactive after oral administration at doses up to 30 mg/kg, a dose 10-fold higher than the minimal effective dose of Compound A.

These results were consistent with those obtained in anaesthetized rats as described in Example 8 above. Compound A was found to be active in increasing the BVC without affecting bladder contractility (MP), in contrast to oxybutynin. Compound A was also found to be active after both i.v. and oral administration, in contrast to NAN-190 which was inactive after i.v. or oral administration of doses up to 10-fold higher than those used for Compound A.

The above results show that compounds endowed with antagonistic activity at pre- and post-synaptic 5-HT_{1A} receptors and devoid of significant affinity for the α_1-adrenergic receptors are unexpectedly endowed with a potent pharmacological activity on the lower urinary tract. In particular, these compounds are able to inhibit the micturition reflex and to increase the period between micturition without impairing the capability of detrusor to have effective voidings once the micturition threshold has been reached. This is important since the drugs currently used for treatment of urinary incontinence (mainly anticholinergics) decrease efficiency of micturition, as shown for oxybutynin in Example 9, when the micturition pressure is reduced, and causes an increase of residual volume, due to compromission of bladder contractile force.

CLAIMS

1. Use of a compound which

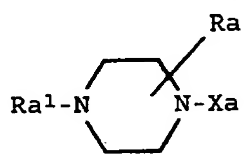
(a) binds to a $5\text{-}HT_{1A}$ receptor with an affinity of at least 10^{-7} M,

(b) binds to a $5\text{-}HT_{1A}$ receptor with an affinity at least 50-fold stronger than the affinity with which the compound binds to an α_1-adrenergic receptor, and

(c) exhibits $5\text{-}HT_{1A}$ receptor antagonist activity on both pre-synaptic and post-synaptic $5\text{-}HT_{1A}$ receptors,

or of a stereoisomer, hydrate, solvate or pharmaceutically acceptable salt of such a compound, for the preparation of a medicament for the treatment of lower urinary tract disorders in mammals.

2. Use according to claim 1 of a compound having the general formula I



(I)

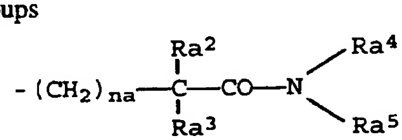
wherein

Ra represents a hydrogen atom or a lower alkyl group;

Ra^1 represents an aryl, nitrogen containing heteroaryl or bicyclic heteroaryl group;

Xa represents one of the groups

(Aa)



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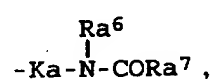

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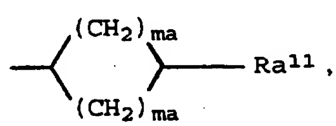

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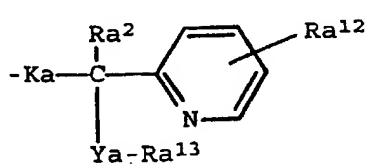

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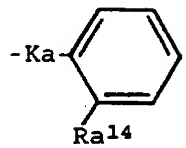
(Ea)  ;

na is 1 or 2;

ma is 1, 2 or 3;

Ra^2 represents a hydrogen atom or a lower alkyl group;

Ra^3 represents an aryl or aryl(lower)alkyl group;

Ra^4 represents a hydrogen atom or a C_1-C_3 alkyl group;

Ra^5 represents a hydrogen atom or a C_1-C_3 alkyl, C_3-C_{12} cycloalkyl or cycloalkyl(lower)alkyl group; or Ra^4 and Ra^5 together with the nitrogen atom to which they are attached represent a 1-azetidinyl, 1-pyrrolidinyl, piperidino, 1-perhydroazepinyl, morpholino, or 1-piperazinyl group, each optionally substituted by a lower alkyl, aryl or aryl(lower)alkyl group;

Ra^6 represents a monocyclic or bicyclic heteroaryl group;

Ra^7 represents a hydrogen atom, a lower alkyl, cycloalkyl, cycloalkenyl, cycloalkyl(lower)alkyl, aryl, aryl(lower)alkyl, heteroaryl or heteroaryl(lower)alkyl group, or a group -NRa^8Ra^9 or ORa^{10};

Ra^8 represents a hydrogen atom or a lower alkyl, aryl or aryl(lower)alkyl group;

Ra^9 represents a hydrogen atom or a lower alkyl, -CO-(lower)alkyl, aryl, -CO-aryl, aryl(lower)alkyl, cycloalkyl or cycloalkyl(lower)alkyl group; or Ra^8 and Ra^9 together with the nitrogen atom to which they are attached represent a saturated heterocyclic group which may contain an additional heteroatom;

Ra^{10} represents a lower alkyl, cycloalkyl, cycloalkyl -(lower)alkyl, aryl, aryl(lower)alkyl, heteroaryl or heteroaryl(lower)alkyl group;

Ra^{11} represents an aryl or nitrogen containing heteroaryl group;

Ra^{12} represents a hydrogen atom or a lower alkyl group;

Ra^{13} represents a hydrogen atom or a C_1-C_3 alkyl, C_3-C_{12} cycloalkyl or cycloalkyl(lower)alkyl group;

Ra^{14} represents an aryl group;

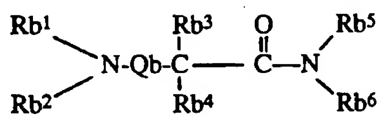
Ka represents a C_2-C_4 alkylene group optionally substituted by one or more lower alkyl groups; and

Ya represents a carbonyl, alkylene, hydroxyalkylene or hydroxycycloalkylene group or a group -$S(O)_{oa}$; where oa = 0 to 2.

3. Use according to claim 1 of 1-[N-cyclohexylcarbonyl-N-(2-pyridyl)- -2-aminoethyl)-4-(2-methoxyphenyl)-piperazine.

4. Use according to claim 1 of 1-[N-cyclohexylcarbonyl-N-(2-pyridyl)--2-aminoethyl)-4-(4-indolyl)-piperazine.

5. Use according to claim 1 of a compound having the general formula II



(II)

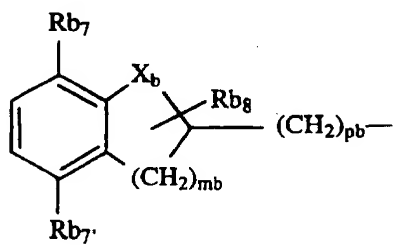
wherein

Qb represents a C_1-C_3 alkylene group, optionally substituted by one or more lower alkyl groups;

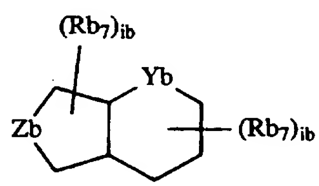
Rb[1] represents a hydrogen atom or a lower alkyl group;

Rb[2] represents one of the groups

(Ab)

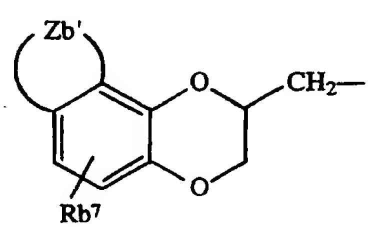


(Bb)



(Cb) Rb[9]-CH_2CH_2- ,

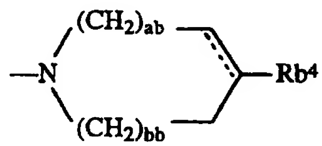
(Db)



(Eb) Rb[10]-O-$CH_2CH(OH)CH_2$- and

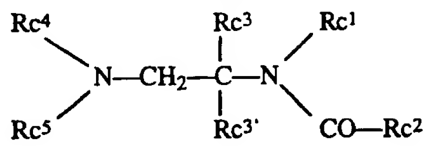
(Fb) Rb[10]-O-CH_2CH_2-;

or Rb^1 and Rb^2 together with the nitrogen atom to which they are attached represent a group of the formula



Rb^3 represents a hydrogen atom or a lower alkyl group;
Rb^4 represents an aryl, bicyclic aryl or heteroaryl group;
Rb^5 represents a hydrogen atom or a lower alkyl group;
Rb^6 represents a hydrogen atom or a C_1-C_{10} alkyl, C_3-C_{12} cycloalkyl, cycloalkyl(lower)alkyl, aryl or aryl(lower)alkyl group;
or Rb^5 and Rb^6 together with the nitrogen atom to which they are attached represent a saturated heterocyclic group, optionally containing an additional hetero atom and optionally substituted by a halogen atom or a lower alkyl, aryl, aryl(lower)alkyl, lower alkoxy or halo(lower)alkyl group;
ab is 0 to 3 and bb is 0 to 3 but (ab + bb) is not more than 3;
---- represents an optional double bond which can be present provided that ab is at least 1;
Xb represents a group $-(CH_2)_{nb}-$, $-OCH_2-$ or $-SCH_2-$;
mb is 0 or 1, nb is 1 to 3, and pb is 0 or 1; provided that (mb + pb) is 1 and (mb + nb) is not more than 3;
Rb^7 represents a hydrogen or halogen atom, or a lower alkyl, (lower)alkylcarbonyl, lower alkoxy, (lower)alkoxycarbonyl, hydroxy, trifluoromethyl, carboxamido, nitro, cyano, amino, (lower)alkylamino or di(lower)alkylamino group;
$Rb^{7'}$ represents a hydrogen or halogen atom; with the proviso that when Xb represents a group $-OCH_2-$ or $-SCH_2-$ then $Rb^{7'}$ represents a hydrogen atom;
Rb^8 represents a hydrogen atom or a lower alkyl group;
ib = 0, 1 or 2; jb = 0, 1 or 2;
Yb represents an oxygen or sulphur atom or a methylene group;
Zb represents the atoms necessary to form a heteroaromatic ring having from 5 to 7 carbon atoms fused to the non-aromatic ring containing the Yb group;
Rb^9 represents a monocyclic or bicyclic heteroaryl group;
Zb' represents either a pair of hydrogen atoms or the atoms necessary to form an aromatic or heteroaromatic ring fused to the benzodioxanyl group; and
Rb^{10} represents a monocyclic or bicyclic aryl or bicyclic heteroaryl group.

6. Use according to claim 1 of a compound having the general formula III



(III)

wherein

Rc^1 represents a heteroaryl or bicyclic heteroaryl group;

Rc^2 represents a cycloalkyl group;

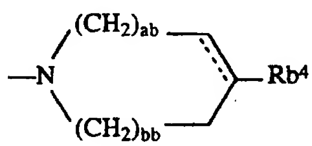
Rc^3 represents a hydrogen atom or a lower alkyl group;

$Rc^{3'}$ represents a hydrogen atom or a lower alkyl group;

Rc^4 represents a hydrogen atom or a lower alkyl group; and

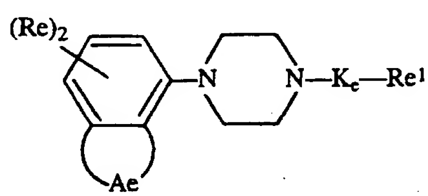
Rc^5 represents one of the groups (Ab), (Bb), (Cb), (Db), (Eb) and (Fb) as defined in claim 5,

or Rc^4 and Rc^5 together with the nitrogen atom to which they are attached represent a group of the formula



wherein ab, bb, Rb^4 and ---- are as defined in claim 5.

7. Use according to claim 1 of a compound having the general formula IV



(IV)

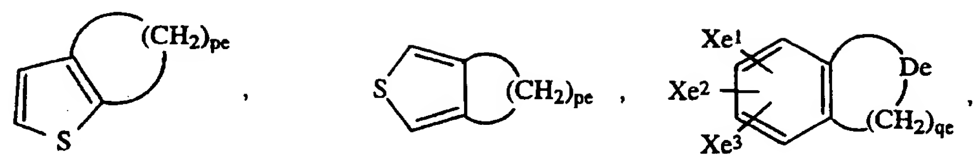
wherein

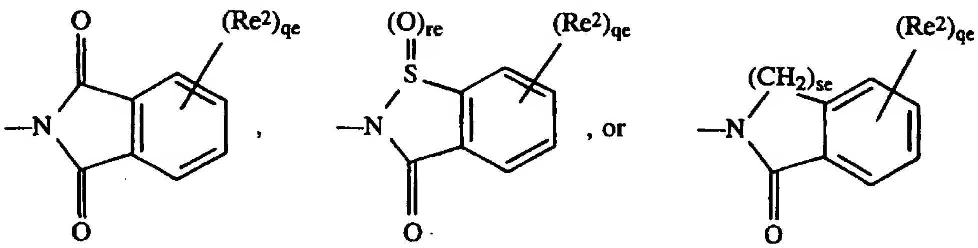
Ae represents a group $-OCH=CH-$, $-OCH_2CH_2-$, $-OCH_2O-$, $-OCH_2CH_2O-$ or $-OCOCH=CH-$;

each Re independently represents a hydrogen or halogen atom or an alkyl, hydroxy, alkoxy, trifluoromethyl or cyano group;

Ke represents a C_1-C_8 linear or branched alkylene group, optionally substituted by an aryl or heteroaryl group;

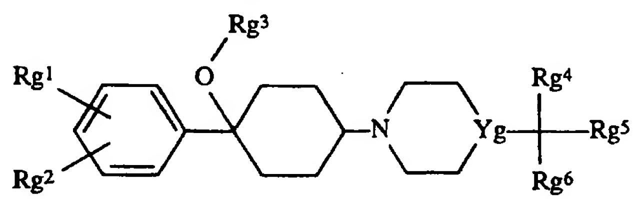
Re^1 represents a phenyl, thienyl, naphthyl or benzothiophenyl group, or a group of the formula





wherein pe is 3 or 4; qe is 0 to 3; re is 0 to 2; se is 1 or 2;
each Re^2 independently represents a halogen atom or an alkyl, hydroxy, alkoxy, trifluoromethyl or cyano group;
De represents a group -CH=CH- or $(CH_2)_{2-4}$; and
each of Xe^1, Xe^2 and Xe^3 independently represents a hydrogen atom or an alkyl, alkoxy, hydroxy, alkylthio, trifluoromethyl, nitro, amino or acetamido group, or two of Xe^1, Xe^2 and Xe^3 together represent a group $-OCH_2O-$ or $-OCH_2CH_2O-$.

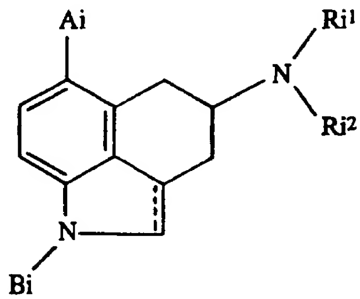
8. Use according to claim 1 of a compound having the general formula V



(V)

wherein
each of Rg^1 and Rg^2 independently represents a hydrogen or halogen atom or a trifluoromethyl or C_1-C_4 alkoxy group;
or Rg^1 and Rg^2, being on adjacent carbon atoms, together represent a group of formula $-O(CH_2)_{ig}O-$ wherein ig is from 1 to 3;
each of Rg^3, Rg^4 and Rg^5 independently represents a hydrogen atom or a C_1-C_4 alkyl group or a phenyl group;
Yg represents a nitrogen atom or a group CH; and
Rg^6 represents a heteroaryl, phenyl or substituted phenyl group.

9. Use according to claim 1 of a compound having the general formula VI



(VI)

wherein

---- represents a single or a double bond;

Ri^1 represents a hydrogen atom, a C_1-C_4 alkyl, C_3-C_4 alkenyl, phenyl(C_1-C_4)alkyl or cyclopropylmethyl group, or a group $CORi^4$, $-(CH_2)_{ni}S(C_1$-$C_4)$alkyl or $-(CH_2)_{ni}C(O)NRi^9Ri^{10}$;

Ri^2 represents a hydrogen atom or a C_1-C_4 alkyl, C_3-C_4 alkenyl or cyclopropylmethyl group;

Ai represents a tetrazolyl or substituted tetrazolyl group, a heteroaryl group having 5 or 6 ring atoms of which from 1 to 3 may be oxygen, sulphur or nitrogen atoms, or a group $Qi{=}\overset{|}{C}{-}Xi$;

Bi represents a hydrogen atom, a C_1-C_4 alkyl group or an amino-blocking group;

Xi represents a hydrogen atom or a group $-ORi^3$, $-SRi^3$ or $-NRi^5Ri^6$;

Ri^3 represents a C_1-C_3 alkyl, substituted C_1-C_3 alkyl, aryl, substituted aryl, aryl(C_1-C_4)alkyl, substituted aryl(C_1-C_4)alkyl or C_3-C_7 cycloalkyl group;

Ri^4 represents a hydrogen atom or a C_1-C_4 alkyl, C_1-C_4 haloalkyl, C_1-C_4 alkoxy or phenyl group;

each of Ri^5 and Ri^6 independently represents a hydrogen atom or a C_1-C_4 alkyl, phenyl(C_1-C_4)alkyl or phenyl group;

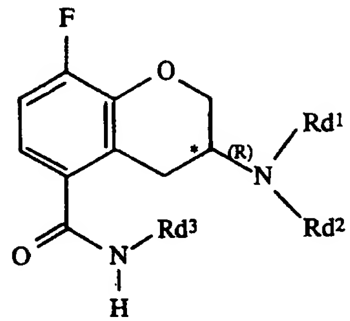
or Ri^5 and Ri^6 together with the nitrogen atom to which they are attached represent a C_3-C_5 heterocyclic ring;

each of Ri^9 and Ri^{10} independently represents a hydrogen atom or a C_1-C_4 alkyl or C_5-C_8 cycloalkyl group;

ni is 1 to 4; and

Qi represents an oxygen or sulphur atom.

10. Use according to claim 1 of a compound having the general formula VII



(VII)

wherein
Rd^1 represents an n-propyl or cyclobutyl group;
Rd^2 represents an isopropyl, t-butyl, cyclobutyl, cyclopentyl or cyclohexyl group; and
Rd^3 represents a hydrogen atom or a methyl group.

Fig.1.

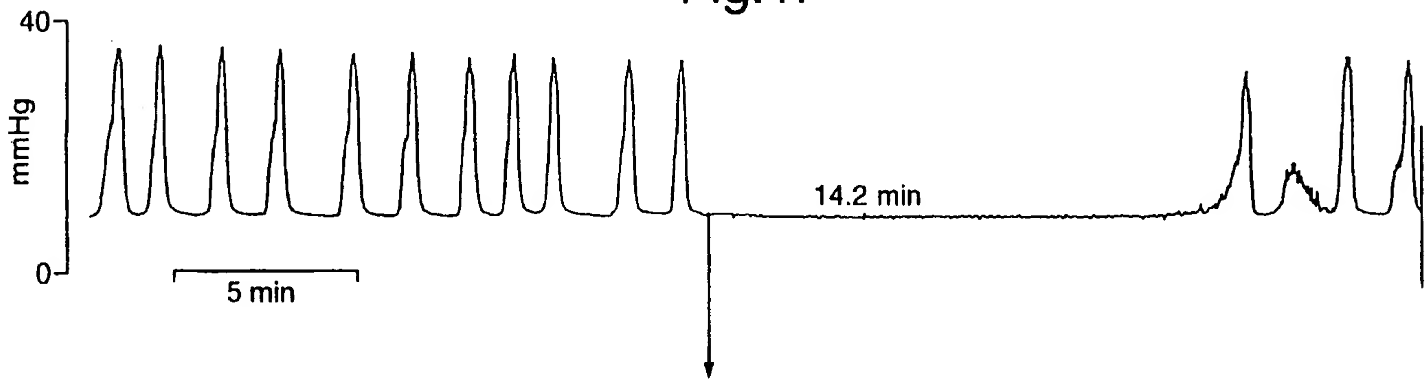


Fig. 1 - Typical tracing showing the effect of Compound A on volume-induced contractions of anaesthetized rats. In the basal period (15 min before the arrow) 9 peaks were recorded. After the i.v. administration of 300 μg/kg of Compound A (at the arrow), 14.2 min of bladder quiescence was observed (disappearance time; no contractions). No change in the height of the peaks was observed.

Fig.2.

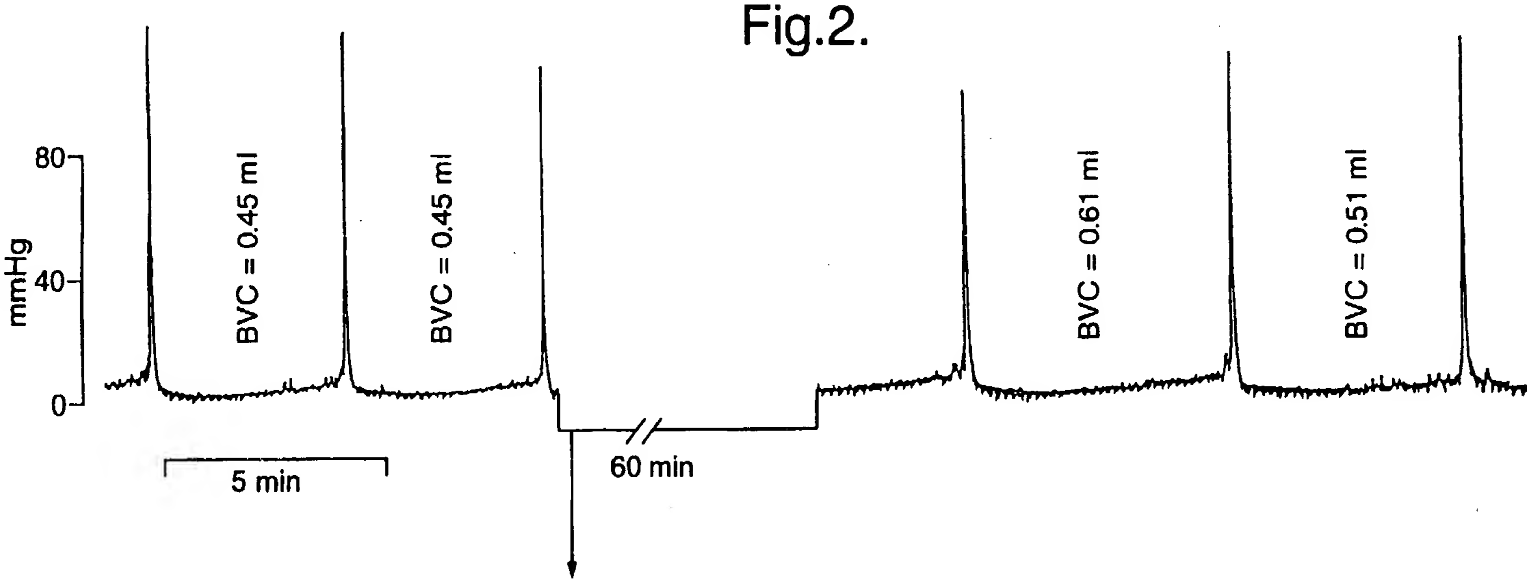


Fig. 2 - Typical tracing showing the effect of Compound A on cystometrographic parameters in conscious rats. Before treatment, two cystometrograms with the same bladder volume capacity (BVC) were recorded. Cystometry was then stopped and the animal was orally treated with 3 mg/kg of Compound A. Cystometrographic recording performed 60 min after the treatment gave two cystometrograms with BVC values of 0.61 and 0.51 ml (35.6 and 13.3% increase, respectively). No substantial changes in micturition pressure were recorded.

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/EP 97/00897

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/495

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 558 245 A (RECORDATI SA) 1 September 1993 see page 6; claims ---	1,10
P,X	WO 96 05817 A (MEDINNOVA) 29 February 1996 see the whole document ---	1-10
A	J.PHARMACOL.EXP.THER., vol. 262, no. 1, 1992, pages 181-189, XP000675356 A. LECCI ET AL.: "Involvement of 5-hydroxytryptamine 1A receptors in the modulation of micturition reflexes in the anaesthetized rat." cited in the application ---	
A	EP 0 512 755 A (WYETH) 11 November 1992 -----	

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
24 June 1997	03.07.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Klaver, T

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 97/00897

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1,2,5-9
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
In view of the large number of compounds which are defined by the wording of the claims, the search has been performed on the general idea and compounds mentioned in the examples of the description.
SEE ANNEX

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 97/00897

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 558245 A	01-09-93	IT 1254469 B	25-09-95
		AU 660067 B	08-06-95
		AU 3377393 A	26-08-93
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		CN 1079738 A	22-12-93
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WO 9605817 A	29-02-96	AU 3263795 A	14-03-96
EP 512755 A	11-11-92	AT 115566 T	15-12-94
		AU 645681 B	20-01-94
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		CA 2067929 A	03-11-92
		CN 1098098 A	01-02-95
		CS 9201344 A	14-10-92
		DE 69200893 D	26-01-95
		DE 69200893 T	13-04-95
		ES 2065133 T	01-02-95
		GB 2255337 A,B	04-11-92
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		IL 101722 A	14-05-96
		JP 5170743 A	09-07-93
		ZA 9203081 A	28-10-93

European Journal of Pharmacology, 213 (1992) 409–415

EJP 52378

Inhibitory effect of inaperisone hydrochloride (inaperisone), a new centrally acting muscle relaxant, on the micturition reflex

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Received 1 August 1991, revised MS received 20 December 1991, accepted 14 January 1992

We examined the effects of inaperisone hydrochloride (inaperisone), a new centrally acting muscle relaxant, on bladder function in anesthetized rats and isolated rat tissues. We also investigated its mechanism of action. When a balloon inserted into the bladder was expanded, rhythmic bladder contractions were observed; inaperisone (4 mg/kg i.v.) abolished these contractions, in both normal and decerebrated rats. The bladder tonus or bladder contraction induced by peripheral stimulation of the pelvic nerve was barely inhibited by inaperisone (4 mg/kg i.v.), but this dose of inaperisone abolished the efferent discharge from the pelvic nerve that accompanied the rhythmic bladder contractions. The doses of intracerebroventricularly (i.c.v.) and intrathecally injected inaperisone which abolished the rhythmic bladder contractions were 10 and 100 μg, respectively. The inhibitory effects of inaperisone (4 mg/kg i.v.) were not diminished by naloxone (1 mg/kg i.v.) or by bicuculline (0.5 mg/kg i.v.), but were diminished by phaclofen (30 mg/kg i.v. or 300 μg i.c.v.). The specific binding of [^{3}H]baclofen to rat brain synaptosomal membranes was barely inhibited by inaperisone (up to 1 mM). From these results, it is speculated that, among other possible mechanisms, inaperisone inhibits the micturition reflex by acting indirectly on $GABA_B$ receptors in the brainstem.

Inaperisone hydrochloride; Micturition reflex; Rhythmic bladder contraction; $GABA_B$ receptors; (Rat)

1. Introduction

Baclofen, a centrally acting muscle relaxant and $GABA_B$ receptor agonist (Hill and Bowery, 1981), is a lipophilic derivative of γ-aminobutyric acid (GABA) which is one of the major inhibitory transmitters in the central nervous system (CNS) (Curtis and Johnston, 1974). Baclofen is clinically useful for the treatment of patients with unstable bladder symptoms (Taylor and Bates, 1979). In such cases, the action of baclofen is thought be due to its influence on spinal (Teague and Merrill, 1978) and brainstem (Kontani et al., 1988) GABA mechanisms.

The recently developed centrally acting muscle relaxant, inaperisone hydrochloride (inaperisone) (fig. 1), has been shown to reduce the severity of decerebrated rigidity in rats (Morikawa et al., 1987). We recently reported that inaperisone also increased bladder capacity in anesthetized rats, cats, and dogs and in conscious rats (Morikawa et al., 1988). In this study, in order to elucidate the actions and mechanism of action of inaperisone on bladder function, we mainly investigated the effects of this drug on rhythmic bladder contractions in anesthetized rats. These contractions were produced by the rhythmic efferent discharge of the pelvic nerve innervating the bladder (De Groat and Ryall, 1969; Sato et al., 1975, 1977, 1980; Morikawa et al., 1989b). In addition, we compared the main actions of inaperisone with those of baclofen, oxybutynin hydrochloride (oxybutynin, an anticholinergic antipollakiuria agent that is clinically useful for the treatment of detrusor instability; Moisey et al., 1980), and atropine sulfate monohydrate (atropine).

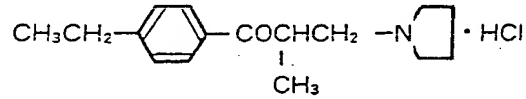

Fig. 1. Chemical structure of inaperisone hydrochloride (inaperisone).

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Applicants: Douglas A. Craig
U.S. Serial No.: 09/450,880
Filed: November 29, 1999
Exhibit 4

2. Materials and methods

2.1. In vivo experiments

Male Wistar rats weighing about 300 g were anesthetized by combined i.p. administration of urethane and α-chloralose at various doses, and were fixed on their backs on a board. The abdomen was opened by a midline incision. The urinary bladder was exposed and a balloon (about 1 ml capacity) was inserted through the apex of the bladder dome. The balloon was connected to a pressure transducer (Nihon Kohden, LPU-0.1) and a syringe with a polyethylene T-tube. The whole system was filled with saline, and variations in intravesical pressure were recorded on a recorder. Both ureters were cut, so that the urine from the kidney was led out of the body. The left jugular vein was cannulated for i.v. injection. For intracerebroventricular (i.c.v.) injection, the rats were anesthetized with pentobarbital (45 mg/kg i.p.). A stainless stylet was implanted, using a stereotaxic technique (Kariya et al., 1982). The stylet was placed in the right ventricle (A: 6.2, L: 1.0, H: +1.2) according to De Groot's stereotaxic atlas (De Groot, 1959). One week after stylet implantation, drugs were injected into the cerebral ventricle with a microsyringe. For intrathecal (i.t.) injection, the drugs were injected into the spinal subarachnoid space (Yaksh and Rudy, 1976). While the animals were under anesthesia, a polyethylene tube was inserted, to a depth of approximately 8.5 cm, through a space between the first and second cerebral vertebrae into the subarachnoid space. The board onto which the rat was fixed was raised by about 1 cm at the head end so that the injected solution could flow caudally. For decerebrated rats, the mid-brain was sectioned with a spatula between the inferior and superior colliculi while the animals were anesthetized.

2.1.1. Rhythmic bladder contractions

Rats were anesthetized with urethane (500 mg/kg i.p.) and α-chloralose (50 mg/kg i.p.), and rhythmic bladder contractions were induced by raising the intravesical pressure (by inflating the inserted balloon) to 10–15 cm H_2O. Drugs were injected after the rhythmic bladder contractions had become stable.

2.1.2. Pelvic or hypogastric nerve activity

For examining pelvic nerve activity, the pelvic nerve of one side and the hypogastric nerves of both sides were divided centrally, at about 5 and 10 mm, respectively, from the pelvic ganglion. For examining hypogastric nerve activity, the hypogastric nerve of one side was divided centrally, at about 10 mm from the pelvic ganglion. The central end of the divided pelvic or hypogastric nerve was isolated from the surrounding connective tissue for as great a distance as possible and was placed on a pair of platinum electrodes and covered with paraffin oil. Efferent discharges from the pelvic or hypogastric nerve were amplified with an amplifier (Nihon Kohden, AVB-10) and displayed on an oscilloscope (Nihon Kohden, VC-10). The oscilloscope display was filmed (Nihon Kohden, RLG-6101). The same discharges were transformed into unit square-wave pulses and these were counted with a pulse counter (Nihon Kohden, AU-601G). As the amplitudes of the efferent discharges were very small and the discharges could not be completely separated from background noise, the trigger level of the pulse counter was adjusted so that it counted only discharges with a voltage higher than an arbitrary threshold. The counts of the pulse counter were recorded on a recorder, together with the rhythmic bladder contractions. Drugs were injected after the efferent discharges had become stable.

2.1.3. Bladder tonus

Rats were anesthetized with urethane (800 mg/kg i.p.) and α-chloralose (80 mg/kg i.p.). Bladder tonus was preserved by raising the intravesical pressure to 10–15 cm H_2O. Drugs were injected after the tonus had become stable.

2.1.4. Bladder contraction induced by stimulation of the peripheral end of the pelvic nerve

Rats were anesthetized with urethane (800 mg/kg i.p.) and α-chloralose (80 mg/kg i.p.). Both pelvic nerves and both hypogastric nerves were divided, at about 5 and 10 mm, respectively, from the pelvic ganglion. One of the peripheral ends of the pelvic nerve was placed on a pair of platinum electrodes and was covered with paraffin oil. The pelvic nerve was stimulated electrically (rectangular pulses of 1-ms duration and supramaximal voltage generated from a stimulator; Nihon Kohden, SEN-3201) for 5 s at 2-min intervals. Drugs were injected after the bladder contractions had become stable.

2.2. In vitro experiment

Male Wistar rats weighing about 300 g were killed by a blow to the back of the head and were exsanguinated. The urinary bladder, excluding the trigone parts, was removed. Longitudinal detrusor strips (5 × 2 mm) were prepared from the posterior and anterior parts of the urinary bladder body. Each detrusor strip was suspended vertically in an organ bath (10 ml) containing Krebs–Henseleit solution continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2 at 37°C. Isometric contractions induced by acetylcholine chloride (ACh, 10^{-3} M) or KCl (60 mM) under a loading tension of 1 g were recorded with a force-displacement transducer (Orientic, T7-30-240). Each strip

was allowed to stand for at least 1 h until its movements became stable. Drugs were administered 30 min before the contractions were induced.

2.3. $GABA_B$ receptor binding

Crude synaptic membranes were prepared from whole brain (Hill and Bowery, 1981). Male Wistar rats weighing about 300 g were killed by a blow to the back of the head and were exsanguinated. The brains were removed and homogenized in 10 volumes of ice-cold 0.32 M sucrose. The homogenate was centrifuged at $1000 \times g$ for 10 min and the supernatant was recentrifuged at $20000 \times g$ for 20 min. The resultant pellet was dispersed in distilled water and centrifuged for 20 min at $8000 \times g$. The supernatant, together with the buffer layer on the pellet, was then centrifuged at $50000 \times g$ for 20 min. The resultant pellet was resuspended in distilled water and again centrifuged at $50000 \times g$ for 20 min. The final pellet was stored at −15°C until used for the radioligand binding assay (at least 16 h). For the assay, the pellet was thawed at room temperature and suspended in 50 mM Tris-HCl buffer containing 2.5 mM $CaCl_2$ (pH 7.4). The suspension was incubated for 45 min at 20°C before being centrifuged at $7000 \times g$ for 10 min. This washing procedure was repeated three more times, allowing a 15-min incubation with each addition of buffer.

The final pellet was resuspended in buffer for the assay. To each 0.4-ml aliquot of membrane suspension (0.2–0.3 mg protein, Bradford, 1976) 0.05 ml of buffer containing drugs and 0.05 ml of buffer containing [^{3}H]baclofen (200 nM) were added. The mixture was incubated for 10 min at 20°C and then centrifuged at $7000 \times g$ for 10 min. The supernatant was aspirated, and the pellet was superficially rinsed with 1 ml of ice-cold buffer and then solubilized with 0.3 ml protosol at 50°C. Radioactivity was measured by liquid scintillation spectrometry (Packard, Model 3385). Specific binding was defined as the difference between bound [^{3}H]baclofen in the presence and absence of 0.1 mM GABA.

2.4. Drugs

Inaperisone and oxybutynin were synthesized in our laboratory. The following drugs were purchased: atropine (Wako), baclofen (Funakoshi), [^{3}H](−)-baclofen (50 Ci/mmol, New England Nuclear), bicuculline methobromide (bicuculline, Funakoshi), GABA (Sigma), morphine hydrochloride (morphine, Dainippon), muscimol hydrobromide (muscimol, Funakoshi), naloxone hydrochloride (naloxone, Sigma) and phaclofen (Funakoshi).

Baclofen and phaclofen were dissolved in saline containing dilute HCl (for in vivo experiments), in distilled water containing dilute HCl (for in vitro experiments) or in 50 mM Tris–HCl buffer containing 2.5 mM $CaCl_2$ (Tris–HCl buffer) (for $GABA_B$ receptor binding). Other drugs were dissolved in saline (for in vivo experiments), in distilled water (for in vitro experiments) or in Tris–HCl buffer (for $GABA_B$ receptor binding). The volumes of solutions injected were 1.0 ml/kg (i.v.), 5 μl (i.c.v.), and 20 μl (i.t.).

2.5. Statistical analysis

Experimental values are expressed as the means ± S.E. Statistical significance was evaluated using Student's t-test.

3. Results

In anesthetized rats, when the intravesical pressure was set between 10–15 cm H_2O by expanding the bladder, using the intravesical balloon with an appropriate amount of water, there were large rhythmical contractions of the bladder (amplitude 50–60 cm H_2O; frequency 0.5–2/min). These contractions were immediately abolished by inaperisone (1–8 mg/kg i.v.) and baclofen (0.3–3 mg/kg i.v.) (fig. 2 and table 1). The effects of inaperisone and baclofen were reversible; both the amplitude and the frequency of the rhythmic bladder contractions returned to pretreatment levels. On the other hand, while the amplitude of the rhythmic bladder contractions was immediately and partially suppressed by oxybutynin (0.01–1 mg/kg i.v.) and atropine (0.01–1 mg/kg i.v.) (fig. 2 and table 1), this effect was not reversible. In addition, oxybutynin (2–8 mg/kg i.v.), like inaperisone and baclofen, also abolished the rhythmic bladder contractions, but the ampli-

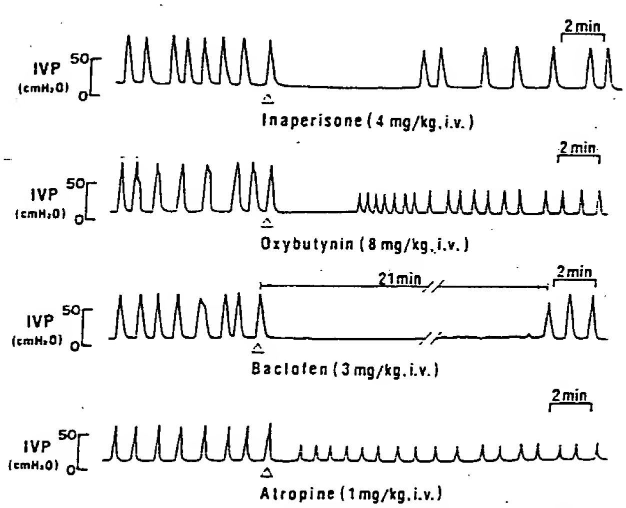

Fig. 2. Effects of various drugs on rhythmic bladder contractions in anesthetized rats.

TABLE 1

Effects of various drugs on rhythmic bladder contractions in anesthetized rats.

Each value represents the mean ± S.E. of three to six rats.

Drug	Dose (mg/kg i.v.)	Disappearance time (min)	Suppression of amplitude (%) [a]
Inaperisone	1	2.1 ± 0.9	15.8 ± 2.5
	2	3.3 ± 1.1 [b]	16.7 ± 5.1 [b]
	4	9.0 ± 1.5 [c]	17.8 ± 5.5
	8	14.4 ± 3.2 [c]	26.3 ± 9.6
Oxybutynin	0.01	< 2	17.8 ± 2.9 [d]
	0.1	< 2	45.1 ± 3.4 [d]
	1	< 2	54.8 ± 3.2 [d]
	2	2.4 ± 1.3 [b]	59.4 ± 4.8 [b,d]
	4	3.8 ± 1.6 [b]	66.8 ± 8.8 [b,d]
	8	5.5 ± 1.5 [b,c]	60.2 ± 2.1 [b,d]
Baclofen	0.3	< 2	3.3 ± 4.4 [b]
	1	11.8 ± 1.0 [c]	4.2 ± 1.3
	3	28.3 ± 1.8 [b,d]	4.5 ± 4.6 [b]
Atropine	0.01	< 2	33.6 ± 6.0 [b,d]
	0.1	< 2	51.3 ± 10.4 [b,d]
	1	< 2	61.9 ± 3.7 [b,d]

[a] Percentage suppression of the amplitude of the rhythmic bladder contractions was determined after the amplitude had recovered, except in cases where the frequency was increased. [b] Value derived from the literature (Morikawa et al., 1988, 1989a). [c,d] Significantly different from the control group (saline) at $P < 0.05$ and $P < 0.01$, respectively.

tude did not return to pretreatment levels (fig. 2 and table 1).

Inaperisone (4 mg/kg i.v.) and baclofen (3 mg/kg i.v.), unlike oxybutynin (8 mg/kg i.v.) and atropine (1 mg/kg i.v.), barely inhibited the bladder tonus or bladder contraction induced by peripheral stimulation of the pelvic nerve in anesthetized rats (table 2).

In addition, inaperisone (10^{-6}–10^{-4} M) and baclofen (10^{-6}–10^{-4} M), unlike oxybutynin (10^{-8}–10^{-5} M) and atropine (10^{-8}–10^{-5} M), barely inhibited the isolated bladder contraction induced by ACh (10^{-3} M) or KCl (60 mM) (fig. 3).

The rhythmic efferent discharges from the pelvic nerve that accompanied the rhythmic bladder contraction in anesthetized rats were abolished by inaperisone (4 mg/kg i.v.) (fig. 4). Similar results were found in two other rats tested. The efferent discharges from the hypogastric nerve that accompanied the rhythmic bladder contractions in anesthetized rats were barely influ-

TABLE 2

Effects of various drugs on bladder tonus and bladder contraction induced by peripheral stimulation of the pelvic nerve in anesthetized rats.

Each value represents the mean ± S.E. of three to seven rats.

Drug	Dose (mg/kg i.v.)	Bladder tonus Change in pressure (cm H_2O)	Bladder contraction (peripheral stimulation of the pelvic nerve) Suppression (%)
Inaperisone	4	0.3 ± 0.2	13.2 ± 4.5
Oxybutynin	8	−3.9 ± 0.7 [b]	39.0 ± 1.3 [b]
Baclofen	3	−1.1 ± 0.4	6.4 ± 2.8
Atropine	1	−1.0 ± 0.6	27.2 ± 5.0 [a]

[a,b] Significantly different from the control group (saline) at $P < 0.05$ and $P < 0.01$, respectively.

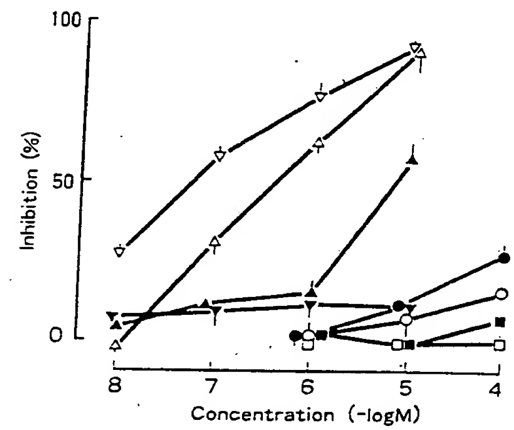

Fig. 3. Effects of inaperisone (○ and ●), oxybutynine (△ and ▲), baclofen (□ and ■) and atropine (▽ and ▼) on isolated bladder contractions induced by ACh (10^{-3} M, open symbol) or KCl (60 mM, closed symbol) in rats. Each point represents the mean ± S.E. of four to eight strips.

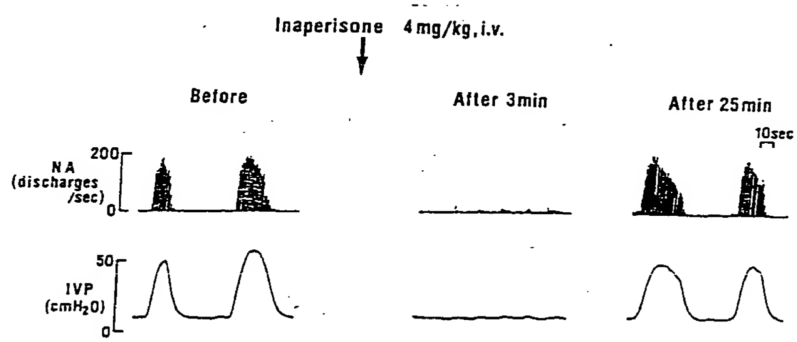

Fig. 4. Effects of inaperisone on rhythmic efferent discharges from the pelvic nerve that accompanied rhythmic bladder contractions in anesthetized rats.

TABLE 3

Effects of i.c.v., i.t. and i.v. injections of inaperisone and baclofen on rhythmic bladder contractions in anesthetized rats. Each value represents the mean ± S.E. of four to six rats.

Drug	Dose (μg) 〈μg/kg〉	Disappearance time (min)			
		i.c.v.	i.t.	i.v.	
				Normal	Decerebrated
Inaperisone	(1)	0.8 ± 0.1	1.3 ± 0.7	–	–
	(10)	5.5 ± 0.7 [b]	1.8 ± 0.8	–	–
	(100)	12.6 ± 1.5 [b]	10.4 ± 1.1 [b]	–	–
	〈4000〉	–	–	9.0 ± 1.5 [b]	9.7 ± 2.5 [b]
Baclofen	(0.001)	1.5 ± 0.5	0.7 ± 0.3	–	–
	(0.01)	6.2 ± 0.8 [b]	3.9 ± 1.2 [b]	–	–
	(0.1)	32.5 ± 4.1 [b]	7.4 ± 0.8 [b]	–	–
	〈3000〉	–	–	28.3 ± 1.8 [a,c]	16.8 ± 2.1 [b]

[a] Value derived from the literature (Morikawa et al., 1989a). [b,c] Significantly different from the control group (saline) at $P < 0.05$ and $P < 0.01$, respectively.

enced by inaperisone (4 mg/kg i.v.) in three rats tested.

The rhythmic bladder contractions in anesthetized rats with an intact CNS were abolished by inaperisone (10 and 100 μg i.c.v.; 100 μg i.t.) and baclofen (0.01 and 0.1 μg i.c.v.; i.t.) (table 3). The rhythmic bladder contractions in anesthetized rats without an intact CNS (intercolliculus-decerebrated rats) were also abolished by inaperisone (4 mg/kg i.v.) and baclofen (3 mg/kg i.v.) (table 3).

The inhibitory effects of inaperisone (4 mg/kg i.v.) on the rhythmic bladder contractions in anesthetized rats were not diminished by pretreatment with naloxone (1 mg/kg i.v.) or bicuculline (0.5 mg/kg i.v.), but were diminished by pretreatment with phaclofen (30 mg/kg i.v.; 300 μg i.c.v.) (figs. 5 and 6). The inhibitory effects of inaperisone (8 mg/kg i.v.; 10 and 100 μg i.c.v.) were also diminished by pretreatment with phaclofen (30 mg/kg i.v.; 300 μg i.c.v.) (figs. 5 and 6). As

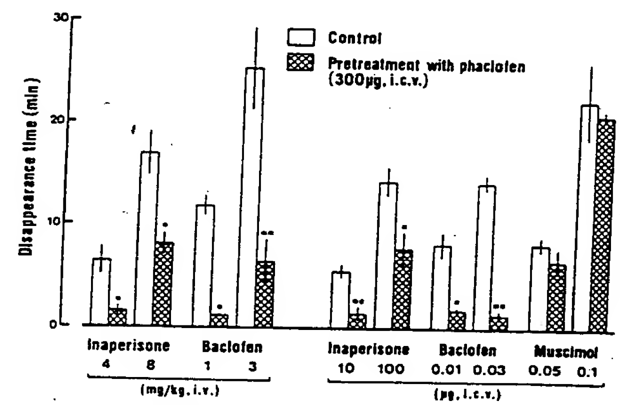

Fig. 6. Effects of pretreatment with phaclofen on the inaperisone-, baclofen- or muscimol-induced inhibition of rhythmic bladder contractions in anesthetized rats. Each bar represents the mean ± S.E. of four to five rats. *, ** Significantly different from the control group at $P < 0.05$ and $P < 0.01$, respectively.

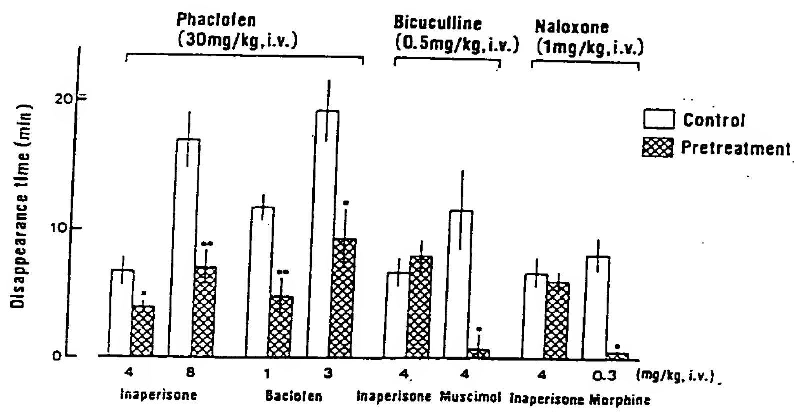

Fig. 5. Effects of pretreatment with phaclofen, bicuculline and naloxone on the inaperisone-induced inhibition of rhythmic bladder contractions in anesthetized rats. Each bar represents the mean ± S.E. of four to six rats. *, ** Significantly different from the control group at $P < 0.05$ and $P < 0.01$, respectively.

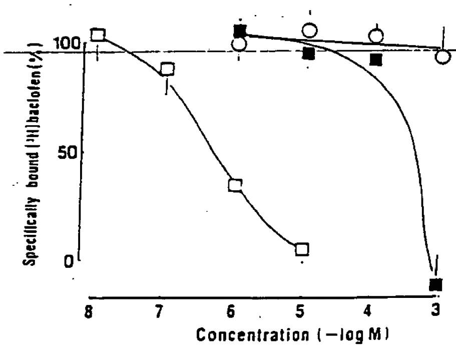

Fig. 7. Displacements of [^{3}H]baclofen from rat brain synaptosomal membranes by inaperisone (○), baclofen (□) and phaclofen (■). Specific binding of 20 mM [^{3}H]baclofen was determined with 100 μM unlabelled GABA. Each point represents the mean of three to five experiments performed in triplicate.

shown in these figures, each dose of naloxone, bicuculline and phaclofen used in this study inhibited the effects of morphine, muscimol and baclofen, respectively. Pretreatment with phaclofen (300 μg i.c.v.) did not inhibit the effects of muscimol (0.05 and 0.1 μg i.c.v.) (fig. 6).

The specific binding of [^{3}H]baclofen to rat brain synaptosomal membranes, unlike that of baclofen (10^{-8}–10^{-5} M) and phaclofen (10^{-6}–10^{-3} M), was barely inhibited by inaperisone (10^{-6}–10^{-3} M) (fig. 7).

4. Discussion

Our results demonstrate that i.v. inaperisone, like baclofen, dose dependently abolished rhythmic bladder contractions in anesthetized rats. The results also show that i.v. inaperisone inhibited the rhythmic efferent discharges of the pelvic nerve innervating the bladder, which are responsible for the production of rhythmic bladder contractions (De Groat and Ryall, 1969; Sato et al., 1975, 1977, 1980; Morikawa et al., 1989b). In addition, the hypogastric vesical nerve input did not contribute to the inaperisone-induced inhibition of the rhythmic bladder contractions. Furthermore, inaperisone, unlike oxybutynin and atropine, did not have an inhibitory effect on the bladder at the smooth muscle level. It is concluded from these results that inaperisone inhibits rhythmic bladder contractions by inhibiting pelvic efferent nerve activity.

The inhibitory effects of inaperisone, like those of baclofen, on these rhythmic bladder contractions are probably of central origin, since the effective dose of inaperisone was 100-fold smaller after i.c.v. than after systemic injection. In addition, the effective dose of inaperisone was 10-fold smaller after i.c.v. than after i.t. injection. The main site of action of inaperisone would thus seem to be the micturition center of the rostral pons in the brainstem (De Groat, 1975). The disappearance of the rhythmic bladder contractions in intercolliculus-decerebrated rats and the rhythmic efferent discharges of the pelvic nerve after injection of inaperisone would therefore result from its action on the micturition center in the brainstem. The precise level of the central action of inaperisone is uncertain at this time and should be investigated further in the near future.

In this study, since the inhibitory effects of inaperisone on the rhythmic bladder contractions were qualitatively similar to those of baclofen, we attempted to examine the effects of pretreatment with certain drugs on the inaperisone-induced inhibition of the bladder contractions. For this investigation, we used phaclofen, a $GABA_B$ receptor antagonist (Kerr et al., 1987), naloxone, an opioid receptor antagonist (Sillén et al. (1985) speculated that the inhibitory effects of baclofen were mediated through interference with opioid mechanisms), and bicuculline, a $GABA_A$ receptor antagonist. The inhibitory effects of inaperisone were not diminished by naloxone or bicuculline, but were diminished by phaclofen. Although relatively large doses of phaclofen (30 mg/kg i.v.; 300 μg i.c.v.) were used in this study, the demonstrated antagonism was not unspecific, since phaclofen itself did not appear to have inherent excitatory properties, nor did it reverse the muscimol ($GABA_A$ receptor agonist)-induced inhibition of the rhythmic bladder contractions. Therefore, it would appear that inaperisone abolished the rhythmic bladder contractions in these anesthetized rats via $GABA_B$ receptors (among other possible mechanisms). Moreover, inaperisone, unlike baclofen or phaclofen, did not inhibit the specific binding of [^{3}H]baclofen to rat brain synaptosomal membranes. From these results, it can be concluded that inaperisone inhibits the micturition reflex by acting indirectly on $GABA_B$ receptors in the brainstem. The indirect mechanism of action of inaperisone is uncertain at present, and further studies are necessary to clarify it.

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EFFECTS OF DRUGS USED IN THE THERAPY OF DETRUSOR HYPERACTIVITY ON THE VOLUME-INDUCED CONTRACTIONS OF THE RAT URINARY BLADDER

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SUMMARY

In this study we examined the effects of the drugs most commonly utilized in the therapy of overactive detrusor, on the volume-induced contractions of rat urinary bladder. Anticholinergics such as propantheline bromide and emepronium bromide, as well as oxybutynin decreased the amplitude of the voiding contractions after intravenous (i.v.) administration in a dose-dependent way. These anticholinergics, on the other hand, generally increased the frequency of the contractions. Nifedipine dose-dependently reduced the amplitude of the contractions. Flavoxate induced a dose-related decrease in the frequency without effects on the amplitude of the peaks. Its main metabolite 3-methylflavone-8-carboxylic acid (MFCA) was inactive after i.v. administration.

Terodiline was active on the amplitude and apparently on the frequency of the voiding contractions. The α-adrenoceptor antagonist prazosin, as well as indomethacin, inhibited only the frequency of the voiding contractions. All the drugs active in reducing the frequency of the voiding contractions after i.v. administration, proved effective also after intracerebroventricular (i.c.v.) injection.

The model of the volume-induced contractions of rat urinary bladder, seems to be a useful tool to evaluate *in vivo* the effects of a compound on the bladder, allowing the possibility of distinguishing among antimuscarinics and calcium antagonists, which peripherally decrease bladder contractility, and other drugs inducing a decrease in the frequency of the voiding reflex acting on the micturition centre(s) in the central nervous system (CNS).

KEY WORDS: rat urinary bladder, voiding contractions, micturition reflex, intracerebroventricular.

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Applicants: Douglas A. Craig
U.S. Serial No.: 09/450,880
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Exhibit 5

INTRODUCTION

In the last decade, disturbances of the function of the lower urinary tract have attracted increasing interest [1]. Drugs with a significant impact on the management of many forms of urinary incontinence and voiding dysfunction are currently utilized, but a long-term selective and side-effect-free treatment is still far from being available, encouraging the search of new substances for the treatment of urinary disturbances [2]. The laboratory studies which have supported the efficacy of many of the drugs commonly used for voiding dysfunction were performed mainly *in vitro* and are affected by the need to extrapolate these data to *in vivo* activity [3]. Adequate *in vivo* animal models are therefore needed for the screening of substances possibly effective in the treatment of urinary incontinence.

In both animals and humans, micturition is initiated and maintained through the activation of a supraspinal vesico-vesical micturition reflex pathway, which can be monitored indirectly by recording the rhythmic, high amplitude intravesical pressure waves which occur when the bladder is distended and maintained under constant volume conditions [4–11].

In this study, we evaluated the usefulness of the volume-induced contractions of rat urinary bladder model in terms of predictivity of drug efficacy, by evaluating the effects of the drugs most commonly utilized in the therapy of overactive detrusor, listed by Andersson [2]. Propantheline bromide and emepronium bromide were chosen as anticholinergics. Flavoxate and nifedipine were studied as examples of drugs with 'direct' muscle effects, and oxybutynin and terodiline as drugs with 'mixed' action (anticholinergic and calcium antagonist). We also studied the effects of prazosin as an α_1-blocking agent and indomethacin as example of prostaglandin synthesis inhibitor, whereas papaverine and morphine were included as a general peripheral antispasmodic drug and a centrally active reference compound, respectively.

MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats (Crl:CD° (SD) BR) from Charles River Italia, weighing 200–250 g, were housed 10 per cage, with food and water *ad libitum* and kept in an animal room at constant temperature of 22°C with a 12 h alternating light-dark cycle, for at least one week before utilization.

Animal preparation and recording

The studies were carried out according to the method reported originally by Dray [4] with some modifications.

Rats were anaesthetized with subcutaneous urethane 1.25 g/kg (5 ml/kg).

For intravenous (i.v.) injection, a PE 50 polyethylene tubing filled with physiological saline was inserted in the jugular vein.

For intracerebroventricular (i.c.v.) administration, a stainless guide was implanted on the skull surface using a stereotaxic technique, 2 mm posterior to Bregma and 2 mm lateral from midline, and drugs were administered in volumes

of 2–20 μl via a Hamilton syringe connected to the guide 4 mm in depth from the skull surface, according to Dray *et al.* [4].

The urinary bladder was catheterized via the urethra by use of PE 50 polyethylene tubing filled with physiological saline.

The catheter was tied in place with a ligature around the external urethral orifice and intravesical pressure was measured by a conventional pressure transducer (Gould-Statham P23ID) and displayed continuously on a chart recorder (Battaglia-Rangoni). The bladder was filled via the recording catheter by incremental volumes of warmed (37°C) saline until spontaneous bladder contractions occurred (usually 0.5–1.0 ml) as a result of central activity. Spontaneous contractions were then recorded and occurred rhythmically and reproducibly for 2–3 h in individual animals. Two parameters were recorded from the cystometrogram: the frequency of voiding contractions calculated by counting the number of peaks/15 min of observation, and the mean amplitude of the contractions (height of the peaks in mmHg).

Drug evaluation

Drug activity, after i.v. administration, was assessed in individual animals against the background of spontaneous bladder contractions by recording the number and height of the peaks for 15 min after drug injection and comparing them with those previously recorded for 15 min after the intravenous administration of the vehicle alone. Drug activity after i.c.v. administration was assessed in a similar way, though pre-injection of vehicle was not performed. However, in control animals (n=20), no changes in frequency and amplitude of the voiding contractions were observed after the i.c.v. administration of the vehicle alone.

Statistical analysis

All the data were reported as mean±SEM.

The statistical significance of the differences 'before' (frequency and amplitude of peaks recorded for 15 min) and 'after' drug administration, was assessed by Student's t-test for paired data [12]. Changes showing a probability P<0.01, were always considered significant.

The all-or-none criterion was introduced to assess the drug activity on frequency and amplitude of the voiding contractions, by considering as positive the treatment-related variations ≥30% of the basal value. Quantal dose-response curves and ED_{50} values were evaluated by the method of Bliss [13], computerized on Wang PC according to the indications reported by Rosiello *et al.* [14].

Drugs

Flavoxate hydrochloride, its main metabolite MFCA (3-methylflavone-8-carboxylic acid) and papaverine hydrochloride, were supplied by Recordati S.p.A., Italy. The other utilized drugs were synthesized in our laboratories or purchased from commercial sources.

For i.v. administration, all the compounds were dissolved in saline with the exception of prazosin (distilled water) and nifedipine (4% *N,N*-dimethyl-formamide and 8% Tween 80 in distilled water). For i.c.v. injection, all the

compounds were dissolved in distilled water with the exception of prazosin that was solubilized with 1% *N,N*-dimethylformamide and 1% Tween 80 in distilled water.

RESULTS

The rapid distension of the urinary bladder in urethane anaesthetized rats produced a series of rhythmic bladder voiding contractions whose characteristics have been described previously [4–7]. By examining the cystometrograms of an elevated number of animals (222) we observed that the mean basal frequency of the voiding contractions was 0.73 peaks/min (11 peaks in 15 min of observation, range 6–24 peaks). The range 8–16 peaks/15 min period was observed in 84% of the animals. The mean value of the amplitude of the basal peaks was 26 mmHg (range 10–47 mmHg).

On the cystometrograms, two different drugs-related effects were generally observed. Some drugs induced a decrease in the amplitude of the pressure peaks, matched as a rule with an increase of their frequency (Fig. 1, upper part). This reduction in amplitude quoted generally no more than 60% of the basal value. At higher doses, the drugs acting on the amplitude of the contractions gave a block of the voidings similar to that reported in the lower part of Fig. 1. When the contractions reappeared, however, their amplitude was lower than that shown in the basal observation period.

Other drugs were active only on the frequency of the voidings, inducing a complete cessation of bladder contractions for a time period generally related to

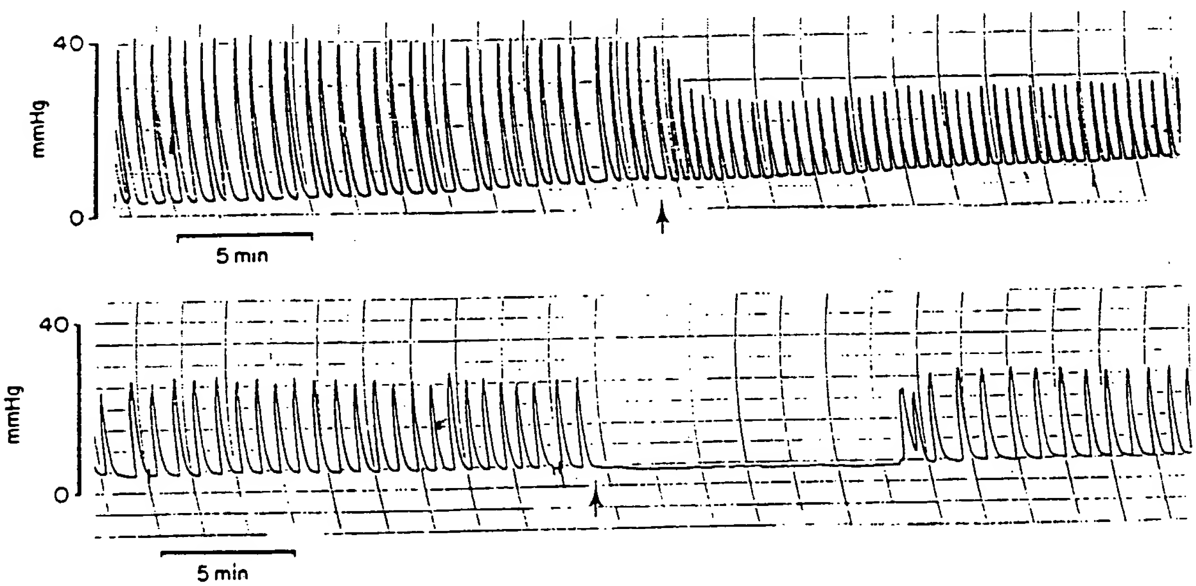

Fig. 1. Typical tracing of the effects after i.v. administration of a drug inducing a decrease of the amplitude and increase of the frequency of the waves (upper trace) or a drug showing a block of the frequency (lower trace), of the voiding contractions. Arrows indicate the drug injection.

the administered dose. When the contractions reappeared, on the other hand, they were of the same amplitude of the basal value (Fig. 1, lower part).

A true reduction of the frequency (increase of the interval between two subsequent peaks) was only sporadically observed, irrespective of the drug administered.

Different methods of evaluation of the effects of the drugs on cystometrogram were investigated. Owing to the maximal reduction observed in the amplitude of the contractions (60%), and to the high variability in the response among animals, we preferred to introduce the quantal criterium reported in the Methods section to evaluate the ED_{50} values on this parameter. This criterion was also utilized to quantify the effect on the frequency of the contractions, though other authors [4, 11, 15] utilized the period time of bladder quiescence to evaluate drug activity. In our experience, the time of quiescence as a parameter for drug evaluation was effective mainly after morphine administration (as reported by Dray [4]) or after i.c.v. administration, but its application was difficult after i.v. injection or when the effects of the other drugs were to be quantitatively assessed. We also tried to quantify the increase of frequency of the voiding contractions, by utilizing naloxone as the reference drug [9–11]. We found that the increase in the number of contractions is a phenomenon rapidly reaching a plateau, and no more than a 30% increase can be observed, both after i.v. and i.c.v. administration (data not shown). We therefore considered as a positive effect a ≥15% increase of the frequency.

The i.v. injection of increasing doses of the two most commonly utilized anticholinergics, propantheline bromide and emepronium bromide, induced a significant and dose-dependent decrease in the amplitude of the contractions (Fig. 2). The maximum decrease observed was 53% after propantheline (0.03 mg/kg) and 47% after emepronium (0.3 mg/kg) injection. By increasing the doses of both compounds, no increase in the percent reduction of the amplitude of the voiding contractions was observed. The number of animals showing more than 30% reduction of peaks amplitude, however, increased after the injection of the highest doses of the drugs.

The number of the peaks, on the other hand, was generally although not significantly increased. The proportion of animals showing a frequency increase more than 15% of the basal value was 4/10, 7/10, 6/10 and 3/5 after propantheline (0.003, 0.01, 0.03 and 0.1 mg/kg, respectively), and 0/7, 3/7, 5/6 and 3/5 after emepronium bromide (0.03, 0.1, 0.3 and 1 mg/kg, respectively). Some animals (four treated with different doses of propantheline and one with 0.03 mg/kg of emepronium) showed a partial block of the voidings, probably due to a complete inhibition of the muscarinic receptors in the bladder, since these animals showed also the highest reduction in the amplitude of the peaks when the rhythmic bladder contractions reappeared.

The drugs classified by K.-E. Andersson [2] as drugs with 'direct' effects, namely flavoxate and nifedipine, behaved differently in this model. Flavoxate induced a dose-related decrease in the frequency of the voiding contractions (Fig. 3), without effects on the amplitude of the peaks. The mean percent inhibition of the number of the peaks observed after the injection of the utilized doses of flavoxate was 4, 15, 39 and 52%, respectively.

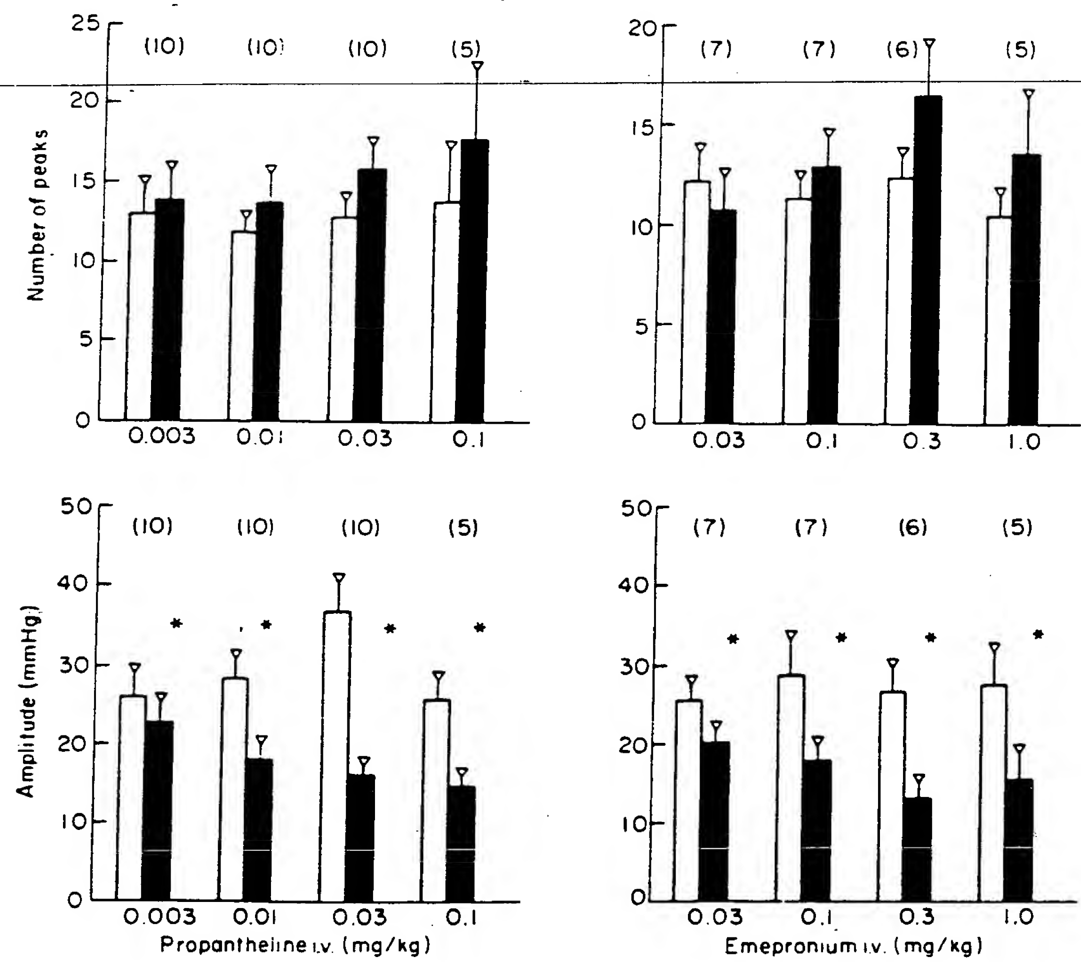

Fig. 2. Effects of different i.v. doses of anticholinergics (propantheline bromide and emepronium bromide) on the frequency (upper part) and the amplitude (lower part) of the rhythmic bladder contractions. Bars represent the mean number of peaks (upper), or the mean height of the peaks (lower) with their SE, recorded 'before' and 'after' drug injection. *=$P<0.01$. The number of animals tested with each dose is reported in brackets. □, Before; ■, after; ▽, SE.

After nifedipine treatment, however, no animals showed a reduction ≥30% in the number of peaks, but four rats out of the ten tested at each dose-level showed frequency increases higher than 15%. Nifedipine dose-dependently decreased the amplitude of the contractions (Fig. 3) and the maximum observed reduction was 37% at 1 mg/kg. Similar effects were obtained by nicardipine administration (data not shown).

Oxybutynin and terodiline are generally reported as drugs with 'mixed' effects, both anticholinergics and calcium antagonists [2, 3]. In the present model, oxybutynin showed effects similar to those of the anticholinergics (Fig. 4). The maximum percent reduction in the amplitude of the voiding contractions was 42% at 1 mg/kg. An increase in the frequency was observed in 2/6 animals treated with the lower doses, and in 6/13 at the highest tested dose. After 0.3 and 1 mg/kg, two rats showed a block of the contractions, owing to the reasons reported above.

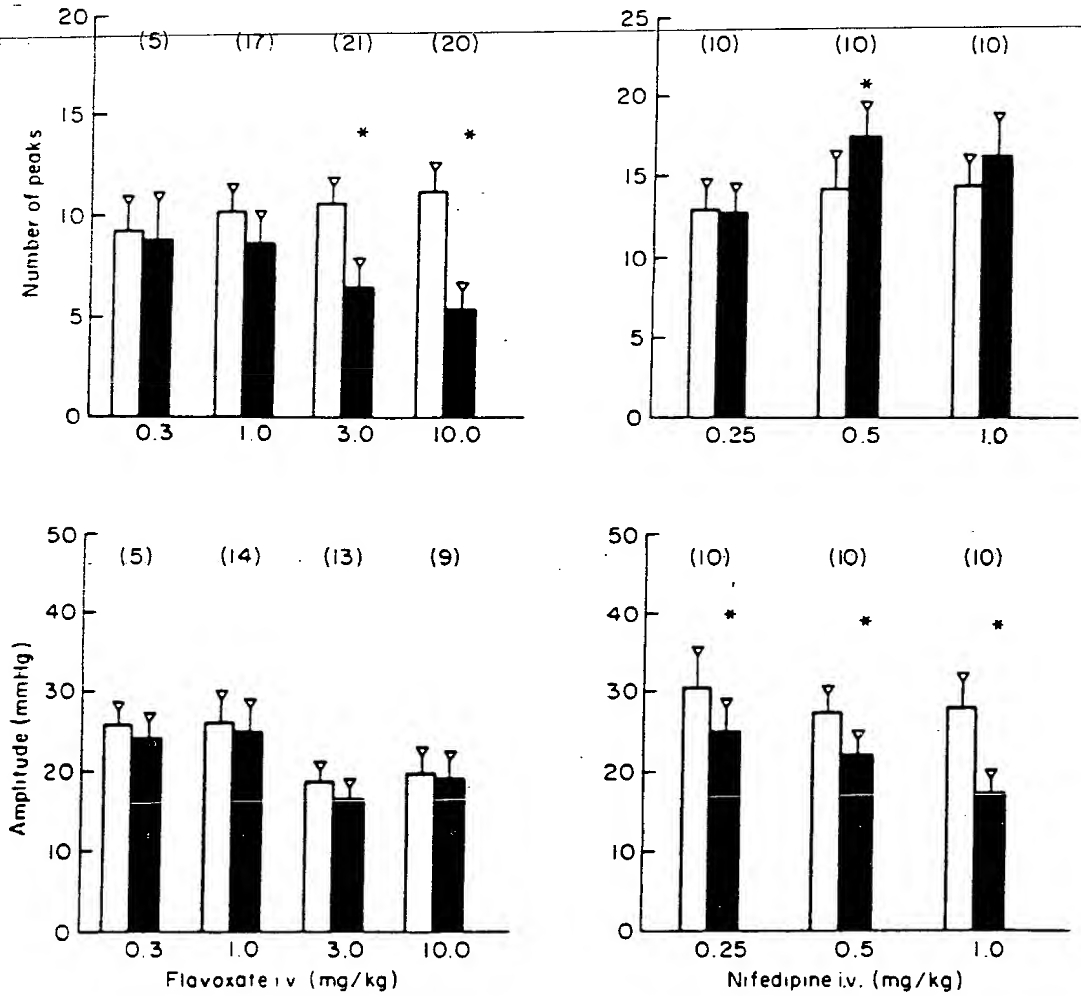

Fig. 3. Effects of different i.v. doses of drugs with 'direct' effects (flavoxate and nifedipine) on the frequency (upper part) and the amplitude (lower part) of the rhythmic bladder contractions. Bars represent the mean number of peaks (upper), or the mean height of the peaks (lower) with their SE, recorded 'before' and 'after' drug injection. $*=P<0.01$. The number of animals tested with each dose is reported in brackets. □, Before; ■, after, ▽, SE.

Terodiline was apparently active both on the frequency and amplitude of the waves (Fig. 4). The maximal observed reduction in amplitude of the voidings was 53%. After the administration of 1 and 3 mg/kg, 2/10 and 3/10 rats respectively, showed an increase ≥15% in the frequency of the voidings. Also, after 3 mg/kg, 3/10 animals showed a block ≥30% of the voidings. It is difficult to ascribe the inhibition of the frequency observed after 10 mg/kg (9/10 treated) to an effect on this parameter or to a block of the muscarinic receptors that induces a complete suppression of the bladder contractions. On the other hand, when the peaks reappeared, their amplitude was generally lower than that shown in the basal observation period, suggesting that the effect of this compound could be similar to that of the antimuscarinics.

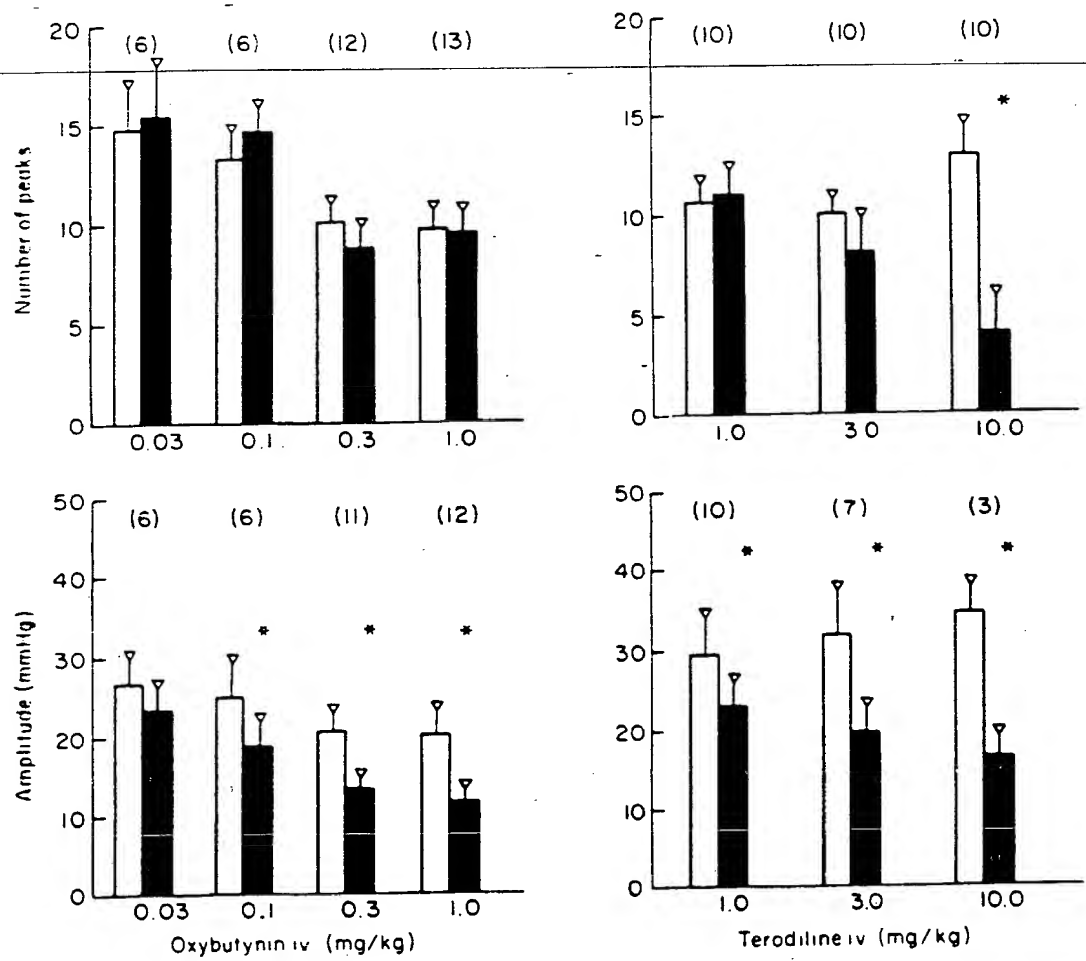

Fig. 4. Effects of different i.v. doses of drugs with 'mixed' actions (oxybutynin and terodiline) on the frequency (upper part) and the amplitude (lower part) of the rhythmic bladder contractions. Bars represent the mean number of peaks (upper), or the mean height of the peaks (lower) with their SE, recorded 'before' and 'after' drug injection. *=P<0.01. The number of animals tested with each dose is reported in brackets. □, Before; ■, after, ▽, SE.

The α-adrenoceptor antagonist prazosin, as well as indomethacin, inhibited only the frequency of the voiding contractions. At the highest tested dose, these two compounds reduced by about 54% the number of peaks observed in the 15 min period after the administration. In Fig. 5, the effects of prazosin and indomethacin are reported.

The effects of papaverine (0.3–3.0 mg/kg), a general antispasmodic drug, and morphine (0.01–0.3 mg/kg), which can be considered the reference drug for this model as reported by Dray *et al.* [4, 9], were also investigated for comparative purposes. Both compounds inhibited only the frequency of the voiding contractions, reaching, at the highest tested dose, 67% and 83% of inhibition, respectively. At these doses, 12/12 and 10/10 animals showed a block ≥30% of the voiding contractions.

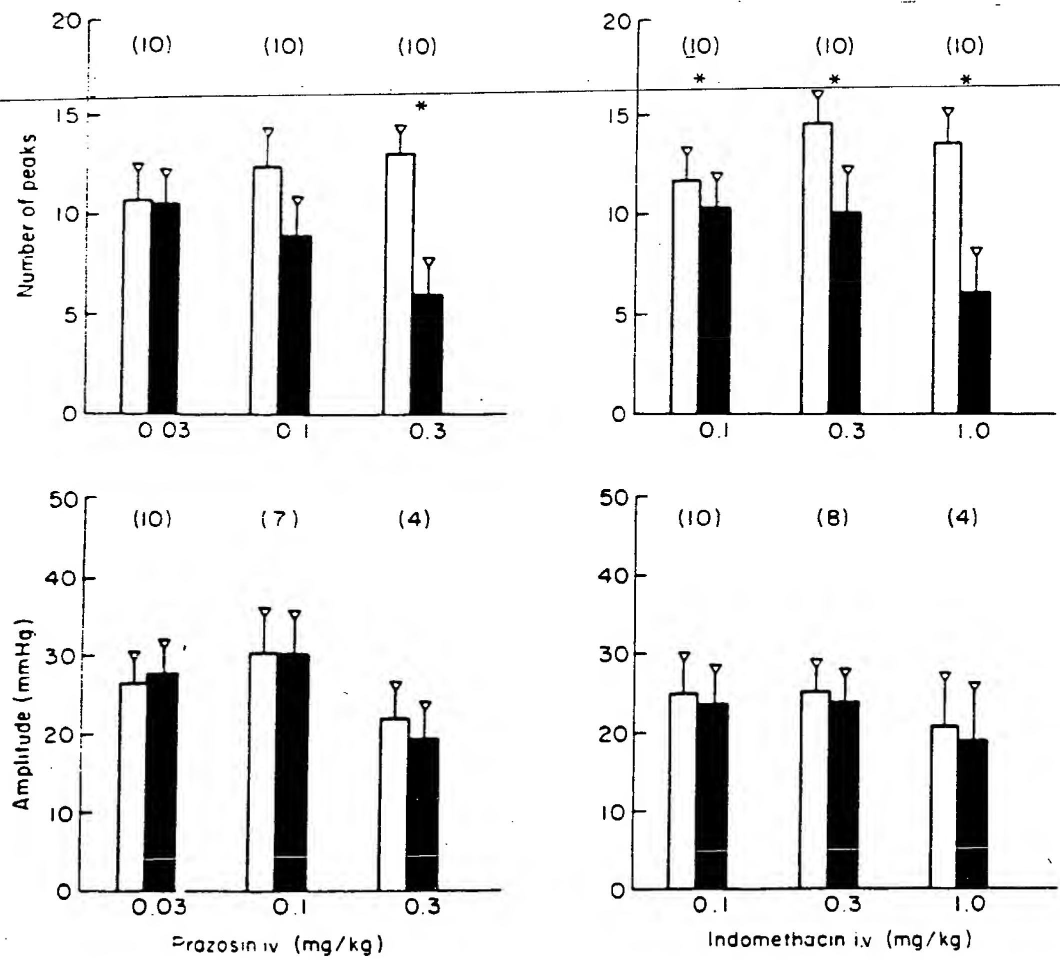

Fig. 5. Effects of different i.v. doses of prazosin and indomethacin, on the frequency (upper part) and the amplitude (lower part) of the rhythmic bladder contractions. Bars represent the mean number of peaks (upper), or the mean height of the peaks (lower) with their SE, recorded 'before' and 'after' drug injection. *=P<0.01. The number of animals tested with each dose is reported in brackets. □, Before; ■, after, ▽, SE.

Since flavoxate is very rapidly metabolized *in vivo* [16] to the corresponding 3-methylflavone-8-carboxylic acid (MFCA), a compound endowed with spasmolytic activity as previously reported [17], we also tested the effects of this metabolite. MFCA was devoid of any activity when injected up to 30 mg/kg. By utilizing the 'all or none' criterion reported in the Methods section, the dose-response curves for the observed effects were constructed. The evaluated ED_{50} values are summarized in Table I.

The drugs acting on the frequency of the voiding contractions, were also tested after i.c.v. administration. All the tested compounds were active after i.c.v. injection, whereas papaverine was devoid of activity up to 300 μg/rat.

Flavoxate (10–300 μg/rat) dose-dependently reduced the frequency of the voiding contractions. After the injection of 100 and 300 μg/rat, this reduction was significant and quoted 39% and 49%, respectively. MFCA was also active after

Table I
***In vivo* effects after i.v. administration of different drugs, on volume-induced contractions of rat urinary bladder.**

	Frequency	*Amplitude*
Propantheline bromide	Increase[a]	0.006 (0.004–0.010)
Emepronium bromide	Increase[a]	0.10 (0.04–0.24)
Flavoxate	2.65 (1.43–4.91)	n.a. up to 10.0
Nifedipine	Increase[a]	0.65 (0.10–4.90)
Oxybutynin	Increase[a]	0.24 (0.14–0.40)
Terodiline	4.35 (2.94–6.45)	2.13 (1.38–3.29)
Prazosin	0.09 (0.06–0.14)	N.a. up to 0.3
Indomethacin	0.51 (0.30–0.88)	N.a. up to 1.0
Papaverine	1.08 (0.81–1.45)	N.a. up to 3.0
Morphine	0.03 (0.02–0.05)	N.a. up to 0.3
MFCA	N.a. up to 30.0	N.a. up to 30.0

[a], no dose-dependent effect; N.a., not active. Data represent the ED_{50} (in mg/kg, and 95% confidence limits) inhibiting the frequency and the amplitude of the contractions.

i.c.v. injection of 30, 100 and 300 μg/rat, and the number of animals showing an inhibition ≥30% was 3/7, 6/10 and 5/10, respectively.

Indomethacin (30–300 μg/rat) and morphine (0.3–3.0 μg/rat) dose-dependently inhibited the voiding contractions. At the highest dose tested, 75% and 78% of inhibition, respectively, was observed.

Prazosin (1–30 μg/rat) was active at low doses, in comparison with flavoxate and indomethacin. A significant reduction in the frequency of the voiding contractions was observed only at the highest tested dose (79%; 6/6 animals showed inhibition ≥30%). However, after 3 and 10 μg/rat injection the number of animals showing an inhibition ≥30% was 4/10 for both doses, and the percent of inhibition of the frequency was 17% and 21%, respectively.

In Fig. 6, the effects of flavoxate, prazosin, indomethacin and morphine are reported as examples. The ED_{50} values calculated for this route of administration are summarized in Table II.

DISCUSSION

The main finding of this study is that drugs with different mechanisms of action can be distinguished by their differing behaviour in this experimental model.

Anticholinergics like propantheline bromide and emepronium bromide, as well as oxybutynin and terodiline, decreased the amplitude of the voiding contractions in a dose-dependent way. This effect may be related to their antimuscarinic activity, as it correlates well with the affinity for bladder muscarinic receptors (*Ki*

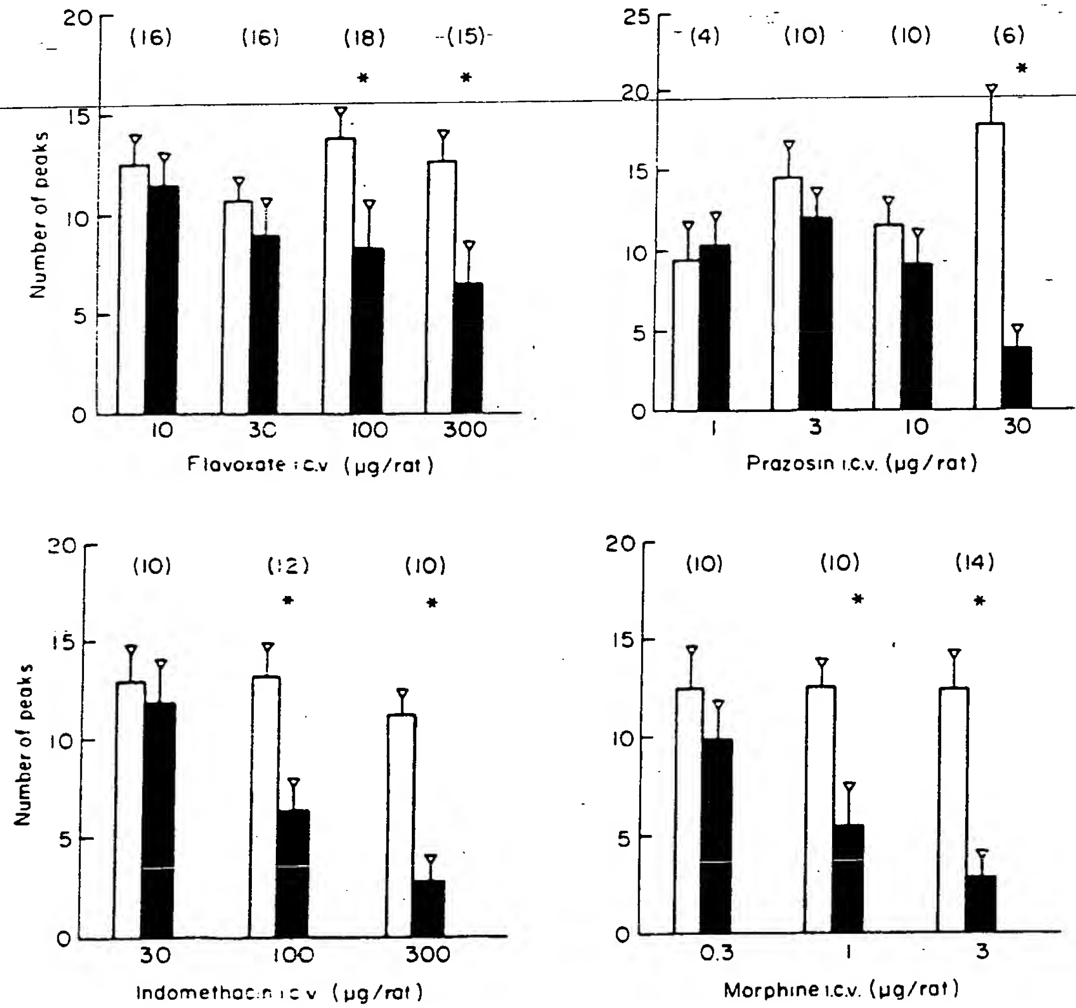

Fig. 6 Effects of different i.c.v. doses of flavoxate, prazosin, indomethacin, and morphine on the frequency of the rhythmic bladder contractions. Bars represent the mean number of peaks with their SE, recorded 'before' and 'after' drug injection. *=P<0.01. The number of animals tested with each dose is reported in brackets. □, Before; ■, after, ▽, SE.

values were 2.1, 27 and 1805 nM for propantheline, oxybutynyn and terodiline respectively [18]). In a recent paper, moreover, Noronha-Blob *et al.* [19] also found a highly significant correlation among the potencies of drugs inhibiting *in vitro* carbachol-induced contractions of isolated guinea pig detrusor muscle and *in vivo* peak intravesical bladder pressure in the guinea pig cystometrogram. The *in vivo* ED_{50} values found by these authors after propantheline, oxybutynin and terodiline administration (0.012; 0.17 and 4.5 mg/kg, respectively), were closely similar to our data. On the other hand, these anticholinergics generally increased the frequency of the voiding contractions. This finding is in agreement with an increase of the frequency and suppression of amplitude of the contractions induced by atropine, as recently reported by Morikawa *et al.* [15].

A block of the muscarinic receptors at the bladder level decreases the efficiency of the cholinergic stimulus originated by the firing of the postganglionic

Table II
***In vivo* effects after i.c.v. administration of different drugs, on the frequency of volume-induced contractions of rat urinary bladder.**

Flavoxate	112 (37–338)
Prazosin	7 (3.6–13.5)
Indomethacin	57 (38–88)
Papaverine	N.a. up to 300
Morphine	0.5 (0.2–1.1)
MFCA	53 (n.d.)

N.a., not active; N.d., not determinable, poor dose-response relationship.
Data represent the ED_{50} in μg/rat, and 95% confidence limits.

efferences, leading to a decrease in the contraction of the detrusor muscle [3]. Similarly nifedipine (and other calcium antagonists), by blocking the influx of extracellular calcium into the muscle cell, shared the effects with the anticholinergics and decreased the amplitude of the voiding contractions.

In contrast to anticholinergics and calcium antagonists, all the other tested drugs (and probably terodiline) induced a decrease or a block of the frequency of the voidings waves without effects on the amplitude of the contractions. Flavoxate and papaverine also behaved in this way, suggesting that in this model the peripheral antispasmodic activity (mainly a calcium-antagonistic activity) claimed as the mechanism of action of these drugs [20, 21], does not play an important role in determining the observed effects. The lack of antimuscarinic properties of flavoxate, which never reduced the pressure peaks' amplitude, was confirmed in this animal model, in agreement with our previously published data [22].

Since a block or a decrease in the frequency of the voiding contractions can be related to effects on the micturition centers [9–11], we also evaluated the activity of these drugs after i.c.v. administration. With the exception of papaverine, all of the drugs were active when directly injected into the left cerebral ventricle.

Morphine was the most active compound, since it has been widely reported that inhibition of bladder contractions is under the control of the endogenous opioid peptides, and that this drug exerts its effects via an interaction with the μ and δ opioid receptors at supraspinal and spinal level [11, 23].

The value of α-adrenoceptor antagonists in the treatment of bladder hyperactivity is still to be established. However, in some patients with benign prostatic hypertrophy treated with phenoxybenzamine or prazosin, bladder instability was reduced or abolished, in addition to the relaxing effects on prostate and urethra [2]. We found that the α-adrenoceptor antagonist prazosin proved potent when injected centrally (in comparison to the other tested drugs, with the exception of morphine). These findings are in agreement with the evidences of an involvement of noradrenaline in the regulation of bladder activity. It has been reported, in fact, that noradrenaline derived from the locus coeruleus activated preganglionic neurons in the sacral intermediolar nuclei via α_1-receptors, thereby producing urinary bladder contraction [24–26].

We found that flavoxate and indomethacin were also active, although at high

doses; after i.c.v. administration, in agreement with the data previously reported by Morikawa *et al.* [27].

Looking at the ratio between the effective doses (ED_{50}) of the tested drugs after i.v. and i.c.v. administration, some considerations on the putative central effect of these drugs can be made.

Morphine gave a ratio of 60, whereas papaverine (that resulted practically inactive when centrally administered) showed a ratio lower than 4, and this value for indomethacin was 9. It is therefore conceivable that these two compounds act mostly at the bladder level directly on the bladder mechanoceptors located in series with the detrusor smooth muscles [6]. Flavoxate gave a ratio of 24, confirming that its action in this model can be related to an activity of the micturition centre, as previously reported [27]. The effects observed after the i.c.v. injection of MFCA, in contrast to its inactivity after i.v. administration, run in the same direction, since flavoxate easily crosses the blood–brain barrier and is rapidly metabolized to MFCA.

In this model, prazosin seems to act mainly as a peripheral agent, showing a ratio of 13. However, it has been reported [28] that it centrally interferes with the somatic control of the lower urinary tract in a different experimental condition.

It is difficult to establish whether or not the different effects observed in the present model can be related to a different clinical efficacy, clinical evaluations often being contradictory. Nevertheless, Cardozo and Stanton [29], reported that only the acute parenteral administrations of emepronium bromide in humans, in contrast to flavoxate and imipramine, caused significant cystometric changes.

Studies have been performed with prostaglandin inhibitors, which provide some symptomatic effects but were not shown to modify cystometry [3].

The administration of α-blockers generally induces a significant improvement in obstructive symptoms (frequency, urgency) such as that elicited by flavoxate [30]. These clinical observations could suggest that drugs acting on the frequency of the voiding contractions can improve the symptomatology joined with detrusor instability.

In conclusion, the discussed model seems to be a useful tool to evaluate *in vivo* the effects of different drugs on the bladder, allowing the possibility of distinguishing between compounds that peripherally decrease the bladder contractility, and other drugs inducing a decrease in the frequency of the voiding reflex via action at the micturition centre(s).

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